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# Aquatic Animal Health Subprogram: Investigation of an emerging bacterial disease in wild Queensland grouper, marine fish and stingrays with production of diagnostic tools to reduce the spread of disease to other states of Australia

**Final report February 2015**

Rachel O. Bowater
FRDC Project No. 2010/034

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Investigation of an emerging bacterial disease in wild Queensland grouper, marine fish and stingrays, with production of diagnostic tools to reduce the spread of disease to other states of Australia. 2010/034.

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# Researcher Contact Details

Name: Rachel O. Bowater
Address: 9-15 Langton Street, Garbutt, QLD 4810

Phone: 07 33304546
Fax: 07 3330 4571
Email: Rachel.bowater@daff.qld.gov.au

# FRDC Contact Details

Address: 25 Geils Court
Deakin ACT 2600
Phone: 02 6285 0400
Fax: 02 6285 0499
Email: frdc@frdc.com.au
Web: www.frdc.com.au

In submitting this report, the researcher has agreed to FRDC publishing this material in its edited form.

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**2010/034 Investigation of an emerging bacterial disease in wild Queensland grouper, marine fish and stingrays with production of diagnostic tools to reduce the spread of disease to other states of Australia**

**PRINCIPAL INVESTIGATOR**: Dr. R. O. Bowater

**ADDRESS:**
Post Office Box 1085
Biosecurity Queensland,
Department of Agriculture, Fisheries & Forestry
Townsville, Queensland, AUSTRALIA 4810
Telephone: 07 3330 4546
Facsimile: 07 3330 4571

**OBJECTIVES:**

1. To create a library of different *Streptococcus agalactiae* strains enabling utilisation by scientific researchers.

2. Develop reliable, rapid and accurate diagnostic tools to enable detection of the bacterial disease streptococcosis caused by *Streptococcus agalactiae* in marine fish.

3. To perform phylogenetic comparison of Australian fish, human and animal strains of *Streptococcus agalactiae* with overseas fish and animal *S. agalactiae* strains, to determine their genetic relatedness and origin of the grouper strain (introduced or endemic).

4. Perform a challenge infectivity trial in Queensland grouper to prove experimentally that *S. agalactiae* causes mortalities in Queensland grouper to fulfil Koch's postulates.

5. To determine the potential food source of infection for Queensland grouper in Trinity Inlet, Cairns.

6. To determine the distribution and prevalence of *S. agalactiae* in fish along the North Queensland coast.

# 1.0 NON TECHNICAL SUMMARY

**OUTCOMES ACHIEVED TO DATE**

This project has assisted in ensuring the future sustainability and profitability of the aquaculture industry and natural fisheries resources in Queensland, Australia (including the Great Barrier Reef Marine Park), by providing industry, the public, State and Commonwealth governments with improved understanding of the occurrence of *Streptococcus agalactiae* in fish and crustaceans in coastal Queensland. Specifically, the project as shown how *S. agalactiae* may be transmitted experimentally in Queensland grouper, the relatedness between Australian *S. agalactiae* strains from animals and humans and has developed diagnostic tools for Australian State Veterinary Laboratories and Universities, that will assist in State and National aquatic animal disease detection, surveillance, disease monitoring and reporting.

The specific outcomes of this project include; 1. Provision of a bacterial strain collection of *S. agalactiae*, for utilization by scientists for further studies on various immunological, microbiological and genetic aspects of *S. agalactiae* from Australia; 2. Provision of evidence on the genetic relatedness of the Queensland grouper *S. agalactiae* strains to other Australian fish, animal and human strains, enabling inference on the origin of the bacterium in Queensland grouper; 3. Provision of four infection models of *S. agalactiae* in fish, demonstrating the pathogenicity and transmission pathways of *S. agalactiae* in juvenile Queensland grouper; 4. Provision of evidence on the prevalence of *S. agalactiae* in a wide range of fish and crustacean species in the Cairns region, and coastal North Queensland.

Knowledge gained from this project will assist in developing biosecurity, health and disease management plans and programs relating to disease control for *S. agalactiae* in aquaculture facilities, commercial marine aquaria and live reef fish holding facilities. The project has resulted in outcomes that support consumer confidence in the safety of Australian seafood as the *S. agalactiae* isolates from fish are genetically different from those isolated from mammals and have never caused disease in terrestrial animals to date. Outcomes of this project have assisted in protecting recreational fisheries, through improved knowledge on prevalence and distribution of *S. agalactiae* within fish species of the Great Barrier Reef Marine Park in Northern Australia.

Queensland grouper, *Epinephelus lanceolatus*, is a protected species under the *Fisheries Regulation 2008*, under the Queensland Fisheries Act 1994. Between 2007 and 2012, 96 wild, adult, giant Queensland grouper were reported dead in Queensland, most occurring in urban coastal regions of Cairns, Port Douglas and Townsville (Bowater *et al*. 2012). Emerging infectious diseases pose a threat to ecosystem biodiversity, and there is increasing evidence supporting the link between human environmental disturbances and emerging infectious diseases of wildlife populations (Daszak *et al*. 2001).

From 2009 to 2012, Queensland Department of Agriculture, Fisheries and Forestry (QDAFF) veterinarians examined many adult (breeding size) Queensland grouper that washed up dead on public beaches or in creeks, and discovered several fish were infected with *Streptococcus agalactiae.* This bacterium had caused bacterial septicaemia and meningitis (brain infection) in numerous wild adult Queensland grouper (Bowater *et al*. 2012). In the same year, sick javelin grunter *Pomadasys kaaken,* catfish *Arius thalassinus,* and a diamond scale mullet *Liza vaigensis* in Trinity inlet, Cairns, were also found infected with *S. agalactiae*. This caused public concern over seafood safety, since grunter are an edible, recreational fish species. In

2009, a disease epizootic occurred at *Sea World,* with hundreds of different species of stingrays dying in '*Ray Reef*', a public display and touch tank pool. Veterinary investigations found the stingrays were infected with *S. agalactiae. Sea World* had recently introduced several wild stingrays into '*Ray Reef*' from North Queensland. The introduced stingrays were originally wild-caught, from the same geographical region of North Queensland, where many Queensland grouper had washed up dead on coastal beaches, and were infected with *S. agalactiae*.

*S. agalactiae* causes neonatal infection in humans and bacterial septicaemia in a wide range of terrestrial animals including horses, cows, dogs, rabbits, guinea pigs, lizards, dolphins, crocodiles, fish, stingrays and bullfrogs (Amborski *et al*. 1983; Domingo *et al*. 1997; Keefe 1997; Schuchat 1998; Hetzel *et al*. 2003; Zappulli *et al*. 2005; Evans, et al. 2006a, 2006b; Bishop *et al*. 2007; Filho *et al*. 2009; Harris *et al*. 2011; Bowater *et al*. 2012; Huang *et al*. 2013; Ren *et al*. 2013). This knowledge fuelled public concern regarding seafood safety. Multiple enquiries to QDAFF and Queensland Health arose from the general public, seafood outlets, recreational, commercial and traditional fishers, state and national media, the Great Barrier Reef Marine Park Authority (GBRMPA), and the Department of Environment and Resource Management (DERM), regarding the zoonotic potential of *S. agalactiae* via human contact with sick dying or dead grouper carcasses on beaches, or from handling or ingestion of other fish species or crustaceans (of unknown disease status) occurring in the vicinity of, or feeding on, grouper carcasses. There was a clear need for more research to answer these questions relating to public health and food safety. Information was also needed to determine the prevalence and distribution of *S. agalactiae* in fish and crustacean populations in the Cairns region, and in other coastal regions of Queensland (many of which are part of the Great Barrier Reef Marine Park) as they are highly valued for recreational fishing, tourism, and general recreation.

Research was needed to determine the potential source of infection for Queensland grouper, since most Queensland grouper were dying in highly urbanized coastal areas, such as Trinity Inlet in Cairns. Trinity Inlet was therefore considered a potential site, for infection for Queensland grouper. Furthermore, overseas studies have showed the confirmed transmission of human pathogenic GBS to wild fish via contaminated sewage. Water discharged into Trinity Inlet includes treated sewer, storm water, and seepage from the town dump. Molecular studies were needed to elucidate the genetic relationships between the different isolates of *S. agalactiae* found in grouper, grunter, catfish, mullet, stingrays, and to compare with the human and other animal strains of *S. agalactiae,* to assist in determining the potential origin of the bacterium and therefore any potential hazard to human health. Possible routes of transmission of the bacterium in wild Queensland grouper were unknown at the time of wild grouper deaths, hence there was a need for research to develop infection models in Queensland grouper, to determine how the bacterium is spread in Queensland grouper, and to fulfill Koch's postulates ie. to provide a complete scientific basis for conclusion that *S. agalactiae* was the pathogen responsible for killing wild Queensland grouper.

In summary, this project achieved all of its objectives. The project demonstrated that *S. agalactiae* is highly pathogenic to juvenile Queensland grouper, *E. lanceolatus* and can be spread via infected water, infected food, by injection, or by cohabitation (of *S. agalactiae*-infected fish with non-infected fish). The project provided a collection of 96 different *S. agalactiae* strains at UQ and QDAFF, from a variety of fish, humans and land animals, to support ongoing scientific research.

This project produced rapid, reliable and accurate diagnostic tools, including PCR and an Immuno-histochemistry (IHC) method to specifically detect *S. agalactiae* in fish, thus increasing the States', Territories', and Australia's capability for disease testing, surveillance, monitoring and reporting. Histopathology with use of special stains also proved to be useful for providing additional diagnostic information.

In spite of targeted surveillance and sampling of over 1300 wild fish and crustaceans *S. agalactiae* was not detected in wild fish and crustaceans in Trinity inlet, Cairns, and other coastal regions of Queensland, between 2010 and 2012. This information, combined with molecular results from this project, gives assurance on seafood safety for fish and crustaceans in Trinity Inlet, Cairns, and coastal Queensland, and the results of this project informed Queenslands risk assessment process.

The project found no evidence to indicate that *S. agalactiae* is present in species commonly used as bait. Sampling of over 200 frozen baitfish and mullet failed to detect *S. agalactiae*. Frozen baitfish also poses a negligible-to-very low risk as a potential source of *S. agalactiae* infection to other marine fish and mammalian marine species that rely on frozen baitfish as a source of food, at commercial marine aquaria, wildlife parks or zoos.

Molecular studies showed the *S. agalactiae* isolates from Queensland grouper were genetically distinct from human, cow, dog, cat or crocodile strains. Molecular studies further showed the Queensland grouper *S. agalactiae* isolates were most closely related to the grunter, mullet, catfish and stingray strains isolated from north Queensland. All North Queensland fish isolates belonged to strain type ST-261, a strain type that has been identified from Nile tilapia and hybrid tilapia species from Indonesia, China, Brazil, Israel and other countries that culture tilapia. Importantly, this sequence type has not been associated with human infection anywhere in the world and is substantially different from all terrestrial isolates of GBS. It is possible that this strain type was imported to Australia with tilapia, or hybrid tilapia species, that were introduced into North Queensland by aquarists over 30 years ago (Arthington *et al*. 1984). This would be consistent with the very high degree of genetic similarity across the whole genome between the Australian fish isolates and tilapia isolates from the US and Israel.

Given the diverse aquatic host range of ST261 GBS, recommendations for further research include the development of biosecurity, health and disease management plans for aquaculture facilities to mitigate any potential transfer from infected wild fish to the aquaculture industry. This is particularly relevant to grouper culture in North Queensland which is on-going and well-established in Cairns, and where farm grow-out trials with several different grouper species are already underway. Other recommendations include the use of infection models for barramundi and other cultured fish species in Australia, to determine their susceptibility to *S. agalactiae.*

This project supports the need for improvements in current State Policy regarding translocation and health testing of wild-caught Australian and imported marine fish, since *S. agalactiae* causes large-scale disease epizootics in aquaculture farms in numerous overseas countries. *S. agalactiae* affects a broad range of marine and freshwater fish, stingrays, saltwater crocodiles and dolphins and therefore poses a threat to Australian native fish species through the inter-state and intra-state movement of subclinical carrier fish and elasmobranch species to the aquaculture industry, the marine aquarium trade, zoos and marine aquaria in Australia. Moreover, *S. agalactiae* may be present in subclinical carrier

fish, as demonstrated through the infection models developed in this project. This highlights the need for careful testing of fish prior to transfer.

**KEYWORDS: Grouper, *Epinephelus lanceolatus*, *Streptococcus agalactiae*, diagnostic test, PCR, FIHC, histology, pathology, aquaculture, meningitis**

# 2.0 ACKNOWLEDGEMENTS

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Numerous people participated in this research project, and these people and their valuable contributions, are listed in Appendix 2, staff.

# 3.0. BACKGROUND

Queensland grouper, *E. lanceolatus*, are a protected species under the *Fisheries Regulation 2008*. Over a period of 6 years, from 2007 to 2012, 96 wild, adult, giant Queensland grouper were reported dead in Queensland. Deaths spanned as far south as the Brisbane River, and as far north as the Daintree River in Northern Queensland. Dead grouper were also reported from the Gulf of Carpentaria, in Weipa, Karumba, and in remote areas North of Karumba, however most deaths were reported from highly urbanised areas of Cairns and Port Douglas, in North Queensland, with peak mortalities occurring in winters of 2008 and 2009 (Bowater *et al*. 2012).

Many dead Queensland grouper had bacterial septicaemia and meningitis, caused by infection with the bacterium *Streptococcus agalactiae* (Bowater *et al*. 2012). This was the first reported case of streptococcosis caused by *S. agalactiae* in wild fish in Queensland, Australia. The disease appears to be a newly emerging bacterial disease of wild Queensland grouper and other marine fish species in Queensland. Emerging infectious diseases may pose a threat to ecosystem biodiversity, and there is growing evidence to support the link between human environmental disturbance and emerging infectious diseases of wildlife populations (Daszak *et al*. 2001).

Natural epizootics of streptococcosis from infection with *S. agalactiae* have occurred in wild mullet (*Liza klunzingeri*) in Kuwait Bay, the Arabian Gulf; in bluefish (*Pomatomus saltatrix*), striped bass (*Morone saxatilis*), and sea trout (*Cynoscion regalis*) in Chesapeake Bay and its tributaries in the USA (Al-Marzouk *et al*. 2005; Baya *et al*. 1990; Evans *et al*. 2002; Gilbert *et al*. 2002; Evans *et al*. 2006a). In Alabama and Florida in the Gulf of Mexico in 1974, a large fish kill involving tens of thousands of dead fish including menhaden (*Brevoortia patronus*), sea catfish (*Arius felis*), striped mullet (*Mugil cephalus*), pinfish (*Lagodon rhomboids*), Atlantic croaker (*Micropogon undulatus*), spot (*Leiostomus xanthurus*), stingray (*Dasyatis* sp.) and silver trout (*Cyonscion nothus*) was attributed to *S. agalactiae* infection (Plumb *et al*. 1974).

*S. agalactiae* has also caused disease epizootics with significant losses in farmed fish; in red tilapia (*Oreochromis* sp.), red hybrid tilapia (*Oreochromis niloticus* x *Oreochromis massambicus*) and nile tilapia (*Oreochromis niloticus*) grown in Indonesia, China, Thailand, Malaysia and Columbia (Direkburakum & Danayadol 1987; Chen *et al*. 2007; Suanyuk *et al*. 2008; Herndadez *et al*. 2009; Lusiastuti *et al*. 2009a,b; Musa *et al*. 2009; Abuseliana *et al*. 2011; Amal *et al*. 2012, 2013; Liu *et al*. 2013; Lusiastuti *et al*. 2014); in gilthead seabream (*Sparus auratus*) and silver promfret (*Pampas argenteus*) in Kuwait (Gilbert *et al*. 2002; Duremdez *et al*. 2004), in ya-fish (*Schizothorax prenanti*) (Geng *et al*. 2012), barcoo grunter (*Scortum barcoo*) a native Australian fish species farmed in China (Liu *et al* 2013), and in golden pompano (*Trachinotus blochii*) in Malaysia (Amal *et al*. 2012).

*S. agalactiae* is a significant pathogen of humans, causing septicaemia and meningitis in human newborn babies and disease in immuno-compromised or elderly humans, with an increasing trend of non-neonatal bacteraemia in older, non-indigenous women in North Queensland over the past 14 years (Domingo *et al*. 1997; Edwards *et al*. 2005; Harris *et al*. 2011). *S. agalactiae* also causes septicaemia in a diverse range of terrestrial and aquatic animals, including horses, cows, monkeys, dogs, cats, guinea pigs, rabbits, crocodiles, dolphins, lizards and bullfrogs (Amborski *et al*. 1983; Miller *et al*. 1996; Keefe 1997;

Lammler *et al*. 1998; Yildirim *et al*. 2002; Hetzel *et al*. 2003; Zappulli *et al*. 2005; Evans *et al*. 2006a; Bishop *et al*. 2007; Ren *et al*. 2013).

In 2009, two sick javelin grunter *Pomadasys kaakan* (Cuvier) found floating, were caught by recreational fishermen in Trinity Inlet, Cairns and confirmed to be infected with *S. agalactiae* (Bowater *et al*. 2012). A sick catfish, *Arius thalassinus* (Ruppell) was also caught in Trinity inlet, Cairns, and was found to be infected with the same bacterium. This caused considerable public concern, since Javelin grunter were a popular edible fish often caught by both recreational and commercial fishers. Hence, the finding of *S. agalactiae* in wild fish resulted in public and industry concern over food safety. The discovery of *S. agalactiae* in edible fish species, along with continuing deaths of wild Queensland grouper in the Cairns region in 2008 and 2009, and deaths occurring in other regional coastal areas of Queensland, attracted considerable public, media and political attention. This included newspaper reports in regional and capital cities of Queensland and attracted national interest in *The Australian*, as well as ABC radio and television.

Following the discovery of the javelin grunter infected with *S. agalactiae*, a small-scale pilot survey of fish was conducted in Trinity Inlet, Cairns by Biosecurity Queensland, targeting six different fish species. Of these, a diamond scale mullet tested positive for *S. agalactiae* (Bowater *et al*. 2012). Mullet are caught locally by recreational bait fishers and are sold fresh or frozen as bait. At commencement of this project it was unknown how many different fish species were infected with *S. agalactiae*, or how prevalent carriage was among fish in coastal waters around urbanised North Queensland where most grouper mortalites occurred. More extensive surveillance studies were therefore required.

At the commencement of this project, *S. agalactiae* had been isolated from a variety of wild fish and elasmobranchs in Queensland including Queensland grouper, mullet, forktail catfish, javelin grunter and four species of wild stingrays (Northern and South-Eastern species), in addition to saltwater crocodiles in the Northern Territory. In each case, the origin of the infection and the strain type was unknown. This was of concern since Queensland has a valuable aquaculture industry including barramundi, prawns and new marine species such as Queensland grouper. The risk of transfer to this industry from wild fish could not be assessed in the absence of evidence of source of infection and possible routes of transmission. Transmission of GBS via water-born exposure has been previously demonstrated in Nile and red tilapia (Mian *et al*. 2009; Rodkhum *et al*. 2011; Abuseliana *et al.* 2011), and transmission through infected prey was implied in crocodile (Bishop *et al*. 2007) and dolphin (Evans *et al*. 2006b). Therefore, investigation of potential routes of transmission was required.

In May 2009, over 40 stingrays died in an epizootic at *Sea World*, a large commercial display aquarium in South East Queensland. Pathology and bacteriology analysis of six estuary rays, *Dasyatis fluviorum* (Ogilby), one mangrove whipray, *Himantura granulata* (Macleay), and one eastern shovelnose ray, *Aptychotrema rostrata* (Shaw) from the outbreak confirmed they died from meningitis and bacterial septicaemia due to infection by *S. agalactiae*. Mortalities included wild-caught stingrays that were translocated from Cairns to *Sea World* (two months prior to the first outbreak) and species sourced from Moreton Bay (South East Queensland). Stingrays are highly valuable, and the total loss to this commercial facility amounted to over $100, 000. There was concern over potential spread to other resident marine animals (sharks and dolphins) at this facility, given the broad host range reported susceptible to *S. agalactiae. S. agalactiae* is present in wild Australian stingrays, and it was speculated whether *S. agalactiae* was transferred from North Queensland to *Sea World* in South East Queensland,

through the translocation of infected, wild-caught stingrays sourced from regions north of Cairns. However this cannot be corroborated or dicarded without accurate typing of the bacterial isolates involved.

Overseas research has shown that certain strains of *S. agalactiae* isolated from mullet and a dolphin in a fish kill in Kuwait were genetically similar to a human pathogenic strain that causes meningitis in newborn babies (Evans *et al*. 2008, 2009).

The findings above highlighted the need for further research, to determine the genetic relatedness, amongst the Australian fish *S. agalactiae* isolates, to find out whether translocation had occurred, and also the relationship between fish and human *S. agalactiae* strains to elucidate any potential risk to human health in consuming or handling wild infected fish. Consequently, two methods were used in the present study for tracing of *S. agalactiae*, multi-locus sequence typing (MLST) and molecular serotyping. MLST was chosen since it does not require physical collection of bacterial reference strains from around the world, but permits accurate comparison with global databases of bacterial isolates online. A public database was established more than ten years ago (Urwin & Maiden, 2003) (pubMLST.org) that is very rich in sequence-typed *S. agalactiae* strains (Jones *et al*. 2003) including many fish isolates (Delannoy *et al*. 2013; Evans *et al*. 2008b). The second method used was molecular serotyping, which explores the genes encoding the surface epitope determinants of the capsular polysaccharide (CPS) (Bentley *et al*. 2006; Honsa *et al*. 2008; Kong *et al*. 2008). CPS operon sequence typing is also physiologically relevant and mutation can be driven quite rapidly by host response, providing local scale resolution amongst isolates (Millard *et al*. 2012).

This project was identified as a high priority by Biosecurity Queensland (QDAFF), the Great Barrier Reef Marine Park Authority (GBRMPA), Queensland Fisheries, Cairns Marine Aquarium, *Sea World*, and was supported by the FRDC Aquatic Animal Health Subprogram. The project had very good support from both commercial and recreational fishers in the Cairns region.

# 4.0. NEED

*S. agalactiae* is an emerging bacterium in wild Australian fish. A challenge model in Queensland grouper, *E. lanceolatus* is required to understand the pathogenesis and mode of transmission of *S. agalactiae* in this fish species. Information generated from experimental challenge trials will be useful for marine aquaculture farmers and marine aquaria to control and manage the disease. It will also assist authorities to develop future biosecurity, health and disease management plans to prevent, control and eradicate the disease. This is needed to mitigate potential disease impacts on aquaculture facilities from the natural environment and fisheries resources, and to ensure sustainability and profitability of aquaculture and other aquatic enterprises.

At the start of this project there were no validated diagnostic tests available in Australia to reliably detect *S. agalactiae* in fish other than bacteriology. Bacterial culture and identification to species level can take several weeks. Development of rapid and accurate diagnostic tools such as PCR and an immuno-histochemistry (IHC) method specific for *S. agalactiae,* are needed to enable rapid, robust diagnosis of *S. agalactiae* in marine fish. The availability of suitable diagnostic tools to detect *S. agalactiae* will have flow-on benefits for all States of Australia, providing them with enhanced detection, diagnostic and surveillance capability for wild and farmed fish.

*S. agalactiae* were isolated from wild deceased Queensland grouper, other species of wild sick and clinically healthy marine fish and wild stingrays from North and South East Queensland by QDAFF veterinarians and microbiologists. There is a need for the development of molecular tests to accurately identify and compare the different strains of *S. agalactiae* in Australian marine grouper and other fish species and elasmobranchs. Strain identification and comparison is also needed to facilitate the potential origin (ie. from human or mammalian origin, or whether native or exotic), determine routes of dissemination of infection during disease epizootics and facilitate vaccine production if needed. Molecular comparative information is also needed to assist with inferring the likely risk of zoonosis (ie. transmission of the bacterium from infected fish to human) via ingestion or handling of infected fish carcases to improve public confidence in consuming Queensland seafood.

Establishment of an Australian collection of *S. agalactiae* isolates at the University of Queensland (UQ) and the QDAFF Biosecurity Sciences Laboratory (BSL) is needed to enable future research on; susceptibility of other Australian cultured fish species; genetic relationships between various Australian and overseas strains; immunology including vaccine development; studies of virulence factors.

The distribution and prevalence of *S. agalactiae* in other wild marine fish besides wild Queensland grouper *E. lanceolatus* is currently unknown. Surveillance studies are needed to determine the distribution and prevalence of *S. agalactiae* in other marine fish, and crustaceans along the eastern coast of Australia, and in the Gulf of Carpentaria, since these locations were where many Queensland grouper were found infected with *S. agalactiae* (Bowater *et al*. 2012). Knowledge on the distribution of various strain types of this newly emerging infectious pathogen is needed by QDAFF Biosecurity, and the GBRMPA, to determine how widespread *S. agalactiae* is in wild populations of fish in north Queensland and to determine the potential threats to the GBRMP fauna, to commercial and recreational fisheries and the tourism industry from this bacterial pathogen. This information is needed to assist; determining whether certain species of fish or crustaceans are a source of infection for

Queensland grouper; determining whether ‘hot spots’ or ‘zones’ for *S. agalactiae* exist in certain geographical locations or regions; to assist with risk management for QDAFF fisheries and for the Great Barrier Reef Marine Park Authority, in relation to the management of the GBR Marine Park where aquaculture enterprises exist.

Bacteriological screening of potential food sources of Queensland grouper for *S. agalactiae* such as frozen and fresh bait fish, and naturally occurring wild fish, crustaceans and elasmobranchs, is needed to determine the potential source of infection for Queensland groupers. Bacterial isolates obtained from wild fish samples or bait will enable molecular comparison to overseas strains to determine whether this pathogen is a newly emerging endemic pathogen or was introduced into Australia.

# 5.0 OBJECTIVES

1. To create a library of different *S. agalactiae* strains enabling utilisation by scientific researchers.

2. Develop reliable, rapid and accurate diagnostic tools to enable detection of the bacterial disease streptococcosis caused by *S. agalactiae* in marine fish.

3. To perform phylogenetic comparison of Australian fish, human and animal strains of *S. agalactiae* with overseas fish and animal *S. agalactiae* strains, to determine their genetic relatedness and origin of the grouper strain (introduced or endemic).

4. Perform a challenge infectivity trial in Queensland grouper to prove experimentally that *S. agalactiae* causes mortalites in Queensland grouper to fulfil Koch's postulates.

5. To determine the distribution and prevalence of *S. agalactiae* in fish along the North Queensland coast

## 6.0 METHODS, RESULTS & DISCUSSION

This Project has ten components, and is therefore divided up as such. The first section 6.1, relates to objective 1 (creating a library of different *S. agalactiae* strains enabling utilisation by scientific researchers). The next three sections; 6.2, 6.3 & 6.4, relate to objective 2 (developing diagnostic test methods to detect *S. agalactiae* in fish). Section 6.5 relates to objective 3 (phylogenetic comparison of Australian fish, human and animal strains of *S. agalactiae* with overseas fish and animal *S. agalactiae* strains, to determine their genetic relatedness and origin of the grouper strain (introduced or endemic)). Sections 6.6 and 6.7 relate to objective 4 (perform a challenge infectivity trial in Queensland grouper to prove experimentally that *S. agalactiae* causes mortalities in Queensland grouper to fulfil Koch's postulates). Sections 6.8, 6.9 and 6.10 relate to objective 5 (determine the distribution and prevalence of *S. agalactiae* in fish along the North Queensland coast), which involved extensive field work, collection and sampling of fish and crustaceans along the coast of Queensland, Australia. For each section, the Methods are described in detail, with Results, a Discussion and a Conclusion presented at the end of each section.

## 6.1 Establishment of a *S. agalactiae* strain collection comprising marine and terrestrial isolates from Australia and International reference collections

### 6.1.1 Methods

Ninety six *S. agalactiae* isolates were collected from a variety of sources and animal species (Table 1) through contacting veterinarians, veterinary schools, aquatic animal health workers, microbiologists and human pathologists. Piscine isolates that were collected included; isolates held at TAAHL from wild Queensland grouper *E. lanceolatus* that had died; isolates from wild fish kill investigations including javelin grunter *Pomadasys kaakan*, mullet *Liza vaigensis*, and catfish *Arius thalassinus*; isolates from several wild marine stingrays that died from two separate epizootics at *Sea World* that were investigated; Human isolates obtained anonymously from Townsville, Northern Queensland; terrestrial animal isolates obtained from sick dogs, cats and cows from Gatton Veterinary School from south-eastern Queensland; estuarine saltwater crocodile *Crocodylus porosus* isolates obtained from Dr. C. Shilton from Berrimah Veterinary Laboratories, Darwin. Due to the prolonged deaths of Queensland grouper occurring along Northern Queensland coastline, bacterial isolates from dead grouper were also gathered as they presented.

Sufficient strains of *S. agalactiae* were collected and established, at both the Tropical & Aquatic Animal Health Laboratory (TAAHL, now defunct), Townsville, and at the University of Queensland (UQ), to allow for molecular and phylogenetic analyses of strains, to meet this Objective.

Bacterial isolates were purified by streaking on Columbia agar base containing 5% defibrinated sheep blood (Oxoid). Strains were then suspended in Todd Hewitt broth, glycerol was added to a final concentration of 20% (v/v), and the resulting suspensions were frozen at -80 °C.

***Confirmation of identity***

Following optimisation of the methodology, bacterial DNA was extracted by enzymatic lysis and phenol chloroform purification. Identity was confirmed by; a) sequencing the 16s rRNA gene using universal primers 29F and 1492R (Table 2) and b) employing a newly optimised PCR based on the AgaF/AdyR primer set (Table 2).

***Polymerase chain reaction (PCR) and sequencing***

Partial length 16s ribosomal DNA from bacterial strains were amplified using DNA polymerase (BioTaq™, Bioline) in 25 µL reactions composed of: 2.5µl 10x $NH_4$ buffer ($Mg^{2+}$ free), 0.75µl $MgCl_2$ (50mM), 0.5µl dNTP (10mM), 0.5µl of each (29F and 1492R) primer (0.2 pmoles/µl (0.2µM)), 0.05µl of Bioline, BioTaq DNA polymerase, 1 µL extracted bacterial DNA (serial 10 x dilution), and the balance made up of sterile Milli-Q water (19.2µl). Amplification conditions were as follows: 3 min at 94°C for one cycle, followed by 30 cycles of denaturation for 15 s at 94°C, 1 min at 48°C (annealing temperature), and extension at 72°C for 2 min. Reaction mixtures were held at 4°C until analysed by agarose gel electrophoresis.

For 16s sequencing, 0.6 µL of Exosapit (Roche Diagnostics) was added to 1.5 µL of PCR product and incubated at 37°C for 30 min followed by heating at 85°C for 15 min. An aliquot (4 µL) of the cleaned product was then combined with 3.5 µL ultra pure water and either

forward or reverse primer (0.6 pmoles $\mu L^{-1}$) before being sent for DNA sequencing by the Australian Genome Research Facility (AGRF).

For specific PCR identification of *S. agalactiae*, DNA templates from unknown bacterial strains were amplified using DNA polymerase (BioTaq™, Bioline) in 25 µL reactions composed of: 2.5µl 10x $NH_4$ buffer ($Mg^{2+}$ free), 0.75µl $MgCl_2$ (50mM), 0.5µl dNTP (10mM), 0.5µl of each (AgaF/AdyR) primer (0.2 pmoles $\mu l^{-1}$ (0.2µM)), 0.05µl of Bioline, BioTaq DNA polymerase, and 1 µL of template encompassing range of concentrations from a serial tenfold dilution of extracted bacterial DNA (above), and the balance made up of sterile Milli-Q water (19.2µl). Amplifications were carried using the following parameters: 3 min at 94°C for one cycle, followed by 30 cycles of denaturation for 15 s at 94°C, 1 min at an annealing temperature across a gradient ranging from 52.9°C to 60°C followed by extension at 72°C for 30s. Reaction mixtures were held at 4°C until analysed by agarose gel electrophoresis.

**Table 2.** Primers used in this study

| Primer | Sequence 5'-3' | Annealing temperature |
|---|---|---|
| 27F | AGAGTTTGATCCTGGCTCAG | 48°C |
| 1492R | GGTTACCTTGTTACGACT | 48°C |
| Aga F | AACAGCCTCGTATTTAAAATGATAGATTAAC | Gradient (53°C – 60°C) |
| Ady R | TCCTACCATGACACTAATGTGTC | Gradient (53°C – 60°C) |

### 6.1.2 Results & Discussion

Ninety six *S. agalactiae* isolates from diverse marine and terrestrial animal hosts were collected from a diverse array of species, from diverse geographical areas, and incorporated into the strain collection at the University of Queensland (UQ) and the Tropical & Aquatic Animal Health Laboratory (TAAHL), QDAFF (Table 1, below). Bacterial isolates of piscine origin included wild sick Queensland grouper, javelin grunter, mullet, catfish, a variety of marine stingrays, human isolates from North Queensland, terrestrial farm animals from South East Queensland, and estuarine saltwater crocodiles from Darwin, the Northern Territory.

A diagnostic PCR was optimised for *S. agalactiae* using the AgaF and AdyR primer pair. An annealing temperature gradient between 52.9 and 60°C resulted in specific amplicons at all temperatures and template concentrations (Fig. 1) The primer pair did not amplify a product from *S. iniae* template at any concentration or temperature (Fig. 1), although a product was obtained from the *S. iniae* template using the Lox 1/Lox2 primer set, confirming the integrity of the control *S. iniae* DNA. Occasionally, the Lox1/Lox2 primer set gave a faint positive with *S. agalactiae* template at 60°C. However, this primer pair is not completely specific to *S. iniae* as originally reported (Mata *et al*. 2004) due to recently identified errors in the Genbank sequence for the *S. iniae* lactate oxidase gene (Nawawi *et al*. 2009). No non-specific reactions were detected with the AgaF/AdyR primer pair under any of the conditions tested with any of the templates tested. Based on these results we recommend use of the AgaF/AdyR primer pair at an annealing temperature 58-60°C using 1-10 ng template DNA for amplification.

All 96 isolates incorporated into the collection were confirmed as *S. agalactiae* either by 16s sequence or subsequently by the specific PCR. 16s sequences were analysed by BLAST against the Genbank database at the NCBI. 16s sequencing, where performed, returned a sequence with 100 % match to *S. agalactiae*. All isolates produced the expected 864 bp

amplicon with the AgaF and AdyR primers. The optimised PCR was transferred to TAAHL for validation.

**Figure 1.** Temperature gradient PCR of *S. agalactiae* and *S. iniae* genomic DNA template using AgaF/AdyR and Lox1/Lox2 primer pairs. AgaF and AgaR are specific for *S. agalactiae*, whilst Lox1/Lox2 are reported specific for *S. iniae*. For each reaction 3µL of PCR product were mixed with 2µL of loading buffer and loaded onto a 1% agarose gel; 5µL of markers (HyperLadder, Bioline).

**Table 1.** *S. agalactiae* strain collection established at UQ and TAAHL, Townsville.

| No. | Genus | species | Host (common name, no.) | Host (species) | year | location | donated by | Tissue / organ of isolation | PCR |
|---|---|---|---|---|---|---|---|---|---|
| 1 | *Streptococcus* | *agalactiae* | Queensland Grouper 1 | *Epinephelus lanceolatus* | 2008 | Cairns | TAAHL | Eye | + |
| 2 | *Streptococcus* | *agalactiae* | Queensland Grouper 1 | *Epinephelus lanceolatus* | 2008 | Cairns | TAAHL | Heart | + |
| 3 | *Streptococcus* | *agalactiae* | Queensland Grouper 2 | *Epinephelus lanceolatus* | 2008 | Cairns | TAAHL | Caudal Kidney | + |
| 4 | *Streptococcus* | *agalactiae* | Queensland Grouper 2 | *Epinephelus lanceolatus* | 2008 | Cairns | TAAHL | Heart | + |
| 5 | *Streptococcus* | *agalactiae* | Queensland Grouper 3 | *Epinephelus lanceolatus* | 2008 | Cairns | TAAHL | Eye | + |
| 6 | *Streptococcus* | *agalactiae* | Javelin grunter 1 | *Pomadasys kaakan* | 2009 | Cairns | TAAHL | Caudal Kidney | + |
| 7 | *Streptococcus* | *agalactiae* | Javelin grunter 1 | *Pomadasys kaakan* | 2009 | Cairns | TAAHL | Eye | + |
| 8 | *Streptococcus* | *agalactiae* | Javelin grunter 1 | *Pomadasys kaakan* | 2009 | Cairns | TAAHL | Operculum | + |
| 9 | *Streptococcus* | *agalactiae* | Catfish 1 | *Arius thalassinus* | 2009 | Cairns | TAAHL | Heart | + |
| 10 | *Streptococcus* | *agalactiae* | Catfish 1 | *Arius thalassinus* | 2009 | Cairns | TAAHL | Brain | + |
| 11 | *Streptococcus* | *agalactiae* | Catfish 1 | *Arius thalassinus* | 2009 | Cairns | TAAHL | Eye | + |
| 12 | *Streptococcus* | *agalactiae* | Mullet | *Liza vaigensis* | 2009 | Cairns | TAAHL | Heart | + |
| 13 | *Streptococcus* | *agalactiae* | Eastern shovelnose ray 1 | *Aptychotrema rostrata* | 2009 | Gold Coast | QML[1] | internal organ | + |
| 14 | *Streptococcus* | *agalactiae* | Mangrove Whiptail ray | *Himantura granulate* | 2009 | Gold Coast | QML[1] | internal organ | + |
| 15 | *Streptococcus* | *agalactiae* | Estuary Ray | *Dasyatis fluviorum* | 2009 | Gold Coast | QML[1] | internal organ | + |
| 16 | *Streptococcus* | *agalactiae* | Estuary Ray | *Dasyatis fluviorum* | 2009 | Gold Coast | QML[1] | internal organ | + |
| 17 | *Streptococcus* | *agalactiae* | Queensland Grouper 4 | *Epinephelus lanceolatus* | 2010 | Townsville | TAAHL | Right eye | + |
| 18 | *Streptococcus* | *agalactiae* | Queensland Grouper 4 | *Epinephelus lanceolatus* | 2010 | Townsville | TAAHL | Kidney | + |
| 19 | *Streptococcus* | *agalactiae* | Queensland Grouper 5 | *Epinephelus lanceolatus* | 2010 | Townsville | TAAHL | Heart | + |
| 20 | *Streptococcus* | *agalactiae* | Queensland Grouper 5 | *Epinephelus lanceolatus* | 2010 | Townsville | TAAHL | Right eye | + |
| 21 | *Streptococcus* | *agalactiae* | Queensland Grouper 5 | *Epinephelus lanceolatus* | 2010 | Townsville | TAAHL | Mandible abscess | + |
| 22 | *Streptococcus* | *agalactiae* | Queensland Grouper 1 | *Epinephelus lanceolatus* | 2008 | Cairns | TAAHL | Eye | + |
| 23 | *Streptococcus* | *agalactiae* | Queensland Grouper 2 | *Epinephelus lanceolatus* | 2008 | Cairns | TAAHL | Head kidney | + |
| 24 | *Streptococcus* | *agalactiae* | Queensland Grouper 3 | *Epinephelus lanceolatus* | 2008 | Cairns | TAAHL | Eye | + |
| 25 | *Streptococcus* | *agalactiae* | Javelin grunter 1 | *Pomadasys kaakan* | 2009 | Cairns | TAAHL | Heart | + |
| 26 | *Streptococcus* | *agalactiae* | Javelin grunter 1 | *Pomadasys kaakan* | 2009 | Cairns | TAAHL | Spleen | + |
| 27 | *Streptococcus* | *agalactiae* | Javelin grunter 1 | *Pomadasys kaakan* | 2009 | Cairns | TAAHL | Pericardium | + |
| 28 | *Streptococcus* | *agalactiae* | Catfish 1 | *Arius thalassinus* | 2009 | Cairns | TAAHL | Caudal kidney | + |
| 29 | *Streptococcus* | *agalactiae* | Catfish 1 | *Arius thalassinus* | 2009 | Cairns | TAAHL | Liver | + |
| 30 | *Streptococcus* | *agalactiae* | Eastern shovelnose ray 1 | *Aptychotrema rostrata* | 2009 | Gold Coast | QML[1] | internal organ | + |
| 31 | *Streptococcus* | *agalactiae* | Eastern shovelnose ray 1 | *Aptychotrema rostrata* | 2009 | Gold Coast | QML[1] | internal organ | + |
| 32 | *Streptococcus* | *agalactiae* | Queensland Grouper 4 | *Epinephelus lanceolatus* | 2010 | Townsville | TAAHL | Spleen | + |
| 33 | *Streptococcus* | *agalactiae* | Queensland Grouper 4 | *Epinephelus lanceolatus* | 2010 | Townsville | TAAHL | Liver | + |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 34 | *Streptococcus* | *agalactiae* | Queensland Grouper 4 | *Epinephelus lanceolatus* | 2010 | Townsville | TAAHL | Left eye | + |
| 35 | *Streptococcus* | *agalactiae* | Human | *Homo sapiens* | 2010 | Townsville | anon | unknown | + |
| 36 | *Streptococcus* | *agalactiae* | dog | *Canis lupis familiaris* | 2005 | unknown | GVS[2] | ear | + |
| 37 | *Streptococcus* | *agalactiae* | dog | *Canis lupis familiaris* | 2008 | unknown | GVS[2] | urine | + |
| 38 | *Streptococcus* | *agalactiae* | cat | *Felis catus* | 2008 | unknown | GVS[2] | Feline draining sinus | + |
| 39 | *Streptococcus* | *agalactiae* | cat | *Felis catus* | 2009 | unknown | GVS[2] | Feline left fore paw | + |
| 40 | *Streptococcus* | *agalactiae* | cow | *Bos taurus* | 2005 | unknown | GVS[2] | Bovine milk | + |
| 41 | *Streptococcus* | *agalactiae* | cow | *Bos taurus* | 2005 | unknown | GVS[2] | Bovine milk | + |
| 42 | *Streptococcus* | *agalactiae* | cow | *Bos taurus* | 2005 | unknown | GVS[2] | Bovine milk | + |
| 43 | *Streptococcus* | *agalactiae* | cow | *Bos taurus* | 2005 | unknown | GVS[2] | Bovine milk | + |
| 44 | *Streptococcus* | *agalactiae* | cow | *Bos taurus* | 2005 | unknown | GVS[2] | Bovine milk | + |
| 45 | *Streptococcus* | *agalactiae* | cow | *Bos taurus* | 2005 | unknown | GVS[2] | Bovine milk | + |
| 46 | *Streptococcus* | *agalactiae* | cow | *Bos taurus* | 2005 | unknown | GVS[2] | Bovine milk | + |
| 47 | *Streptococcus* | *agalactiae* | cow | *Bos taurus* | 2006 | unknown | GVS[2] | Bovine milk | + |
| 48 | *Streptococcus* | *agalactiae* | cow | *Bos taurus* | 2009 | unknown | GVS[2] | Bovine milk | + |
| 49 | *Streptococcus* | *agalactiae* | cow | *Bos taurus* | 2009 | unknown | GVS[2] | Bovine milk | + |
| 50 | *Streptococcus* | *agalactiae* | cow | *Bos taurus* | 2009 | unknown | GVS[2] | Bovine milk | + |
| 51 | *Streptococcus* | *agalactiae* | cow | *Bos taurus* | 2009 | unknown | GVS[2] | Bovine milk | + |
| 52 | Streptococcus | *agalactiae* | Estuary Ray 5 | *Dasyatis fluviorum* | 2010 | Gold Coast | QML[1] | Brain | + |
| 53 | Streptococcus | *agalactiae* | Estuary Ray 6 | *Dasyatis fluviorum* | 2010 | Gold Coast | QML[1] | Oviduct | + |
| 54 | Streptococcus | *agalactiae* | Estuary Ray 7 | *Dasyatis fluviorum* | 2010 | Gold Coast | QML[1] | Heart | + |
| 55 | Streptococcus | *agalactiae* | Estuary Ray 7 | *Dasyatis fluviorum* | 2010 | Gold Coast | QML[1] | Spleen | + |
| 56 | Streptococcus | *agalactiae* | Estuary Ray 7 | *Dasyatis fluviorum* | 2010 | Gold Coast | QML[1] | Liver | + |
| 57 | Streptococcus | *agalactiae* | Estuary Ray 8 | *Dasyatis fluviorum* | 2010 | Gold Coast | QML[1] | Brain | + |
| 58 | Streptococcus | *agalactiae* | Estuary Ray 8 | *Dasyatis fluviorum* | 2010 | Gold Coast | QML[1] | Liver | + |
| 59 | Streptococcus | *agalactiae* | Estuary Ray 8 | *Dasyatis fluviorum* | 2010 | Gold Coast | QML[1] | Spleen | + |
| 60 | Streptococcus | *agalactiae* | Estuary Ray 8 | *Dasyatis fluviorum* | 2010 | Gold Coast | QML[1] | Heart | + |
| 61 | Streptococcus | *agalactiae* | Estuary Ray 8 | *Dasyatis fluviorum* | 2010 | Gold Coast | QML[1] | Kidney | + |
| 62 | Streptococcus | *agalactiae* | Estuary Ray 8 | *Dasyatis fluviorum* | 2010 | Gold Coast | QML[1] | Oviduct | + |
| 63 | *Streptococcus* | *agalactiae* | Crocodile 16 | *Crocodylus porosus* | 2010 | Darwin | BVL[3] | leg | + |
| 64 | *Streptococcus* | *agalactiae* | Crocodile 1 | *Crocodylus porosus* | 2010 | Darwin | BVL[3] | body wall | + |
| 65 | *Streptococcus* | *agalactiae* | Crocodile 2 | *Crocodylus porosus* | 2010 | Darwin | BVL[3] | neck ulcer | + |
| 66 | *Streptococcus* | *agalactiae* | Crocodile | *Crocodylus porosus* | 2010 | Darwin | BVL[3] | left hind leg | + |
| 67 | *Streptococcus* | *agalactiae* | Crocodile | *Crocodylus porosus* | 2010 | Darwin | BVL[3] | right leg | + |
| 68 | *Streptococcus* | *agalactiae* | Crocodile | *Crocodylus porosus* | 2010 | Darwin | BVL[3] | joint | + |
| 69 | *Streptococcus* | *agalactiae* | Crocodile 1 | *Crocodylus porosus* | 2010 | Darwin | BVL[3] | lung | + |
| 70 | *Streptococcus* | *agalactiae* | Crocodile 1 | *Crocodylus porosus* | 2010 | Darwin | BVL[3] | spleen | + |
| 71 | *Streptococcus* | *agalactiae* | Crocodile 1 | *Crocodylus porosus* | 2010 | Darwin | BVL[3] | skin lesion | + |

| 72 | *Streptococcus* | *agalactiae* | Crocodile | *Crocodylus porosus* | 2010 | Darwin | BVL[3] | skin | + |
|---|---|---|---|---|---|---|---|---|---|
| 73 | *Streptococcus* | *agalactiae* | Crocodile | *Crocodylus porosus* | 2010 | Darwin | BVL[3] | liver | + |
| 74 | *Streptococcus* | *agalactiae* | Crocodile | *Crocodylus porosus* | 2010 | Darwin | BVL[3] | lung | + |
| 75 | *Streptococcus* | *agalactiae* | Crocodile | *Crocodylus porosus* | 2010 | Darwin | BVL[3] | liver | + |
| 76 | *Streptococcus* | *agalactiae* | Crocodile | *Crocodylus porosus* | 2010 | Darwin | BVL[3] | spleen | + |
| 77 | *Streptococcus* | *agalactiae* | Crocodile | *Crocodylus porosus* | 2010 | Darwin | BVL[3] | skin | + |
| 78 | *Streptococcus* | *agalactiae* | Estuary Ray 7 | *Dasyatis fluviorum* | 2010 | Gold Coast | QML[1] | Brain | + |
| 79 | *Streptococcus* | *agalactiae* | Estuary Ray 7 | *Dasyatis fluviorum* | 2010 | Gold Coast | QML[1] | Kidney | + |
| 80 | *Streptococcus* | *agalactiae* | Human | *Homo sapiens* | 2011 | Townsville | anon | Female genital | + |
| 81 | *Streptococcus* | *agalactiae* | Human | *Homo sapiens* | 2011 | Townsville | anon | Female genital | + |
| 82 | *Streptococcus* | *agalactiae* | Human | *Homo sapiens* | 2011 | Townsville | anon | Female genital | + |
| 83 | *Streptococcus* | *agalactiae* | Human | *Homo sapiens* | 2011 | Townsville | anon | Female genital | + |
| 84 | *Streptococcus* | *agalactiae* | Human | *Homo sapiens* | 2011 | Townsville | anon | Female genital | + |
| 85 | *Streptococcus* | *agalactiae* | Human | *Homo sapiens* | 2011 | Townsville | anon | Female genital | + |
| 86 | *Streptococcus* | *agalactiae* | Human | *Homo sapiens* | 2011 | Townsville | anon | Female genital | + |
| 87 | *Streptococcus* | *agalactiae* | Human | *Homo sapiens* | 2011 | Townsville | anon | Foot | + |
| 88 | *Streptococcus* | *agalactiae* | Human | *Homo sapiens* | 2011 | Townsville | anon | Male penile | + |
| 89 | *Streptococcus* | *agalactiae* | Human | *Homo sapiens* | 2011 | Townsville | anon | Female genital | + |
| 90 | *Streptococcus* | *agalactiae* | Human | *Homo sapiens* | 2011 | Townsville | anon | Female genital | + |
| 91 | *Streptococcus* | *agalactiae* | Human | *Homo sapiens* | 2011 | Townsville | anon | Female genital | + |
| 92 | *Streptococcus* | *agalactiae* | Human | *Homo sapiens* | 2011 | Townsville | anon | Female genital | + |
| 93 | *Streptococcus* | *agalactiae* | Human | *Homo sapiens* | 2011 | Townsville | anon | Female genital | + |
| 94 | *Streptococcus* | *agalactiae* | Human | *Homo sapiens* | 2011 | Townsville | anon | Female genital | + |
| 95 | *Streptococcus* | *agalactiae* | Queensland Grouper 6 | *Epinephelus lanceolatus* | 2010 | Gulf of Carpentaria | TAAHL | eye | + |
| 96 | *Streptococcus* | *agalactiae* | Queensland Grouper 7 | *Epinephelus lanceolatus* | 2011 | Townsville | TAAHL | eye | + |

[1.] Queensland Medical Laboratories, Vetnostics. Murrarie, Queensland, Australia
[2.] Gatton Veterinary School, Gatton, Queensland, Australia.
[3.] Berrimah Veterinary Laboratories, Northern Territory Government, Darwin, Northern Territory, Australia.

## 6.2 Validation of the diagnostic PCR on cultured bacteria

### 6.2.1 Methods

The PCR to detect *S. agalactiae* using the specific primer pair (AgaF/AdyR) was validated for diagnostic use on bacterial cultures at TAAHL. Validation of the assay was done according to the general outline provided by the World Organisation for Animal Health (OIE, 2013) and Subcommittee on Animal Health Laboratory Standards (SCAHLS). Note that SCAHLS and the OIE outlines do not provide definitive details of the number of cultures to test for validation of an assay. Briefly the PCR method developed at UQ (herein referred to as the PCR assay) was transferred to TAAHL, and its effectiveness as a diagnostic tool demonstrated with respect to specificity, sensitivity, repeatability and robustness.

***Specificity*** refers to the ability of the assay to positively detect *S. agalactiae* and not react positively with other non-*S. agalactiae* agents such as other bacterial species or components of fish tissue. To demonstrate specificity, tissues which are confirmed to be positive or negative for *S. agalactiae* by '*gold standard techniques*', are required. '*Gold standard techniques*' in the detection of *S. agalactiae* in tissues, include bacterial isolation and histopathology. Recently, *in-situ* hybridisation assays have also been used to verify the results of PCR analysis. Specificity of a PCR assay, beyond primer design, is usually achieved by the process of "optimisation" whereby sample extraction, PCR mix reagents and cycling conditions are modified to remove non-specific positive reactors, while maximising detection of known positives.

***Sensitivity*** refers to the lowest concentration of *S. agalactiae* which can be positively detected by the assay. In order to determine sensitivity, *S. agalactiae* positive tissues across a high to low concentration range are required and confirmed by detection with a "*gold standard technique*". Such tissues can be obtained by "spiking" negative tissues with *S. agalactiae* or by using tissues which are confirmed as naturally or experimentally infected with *S. agalactiae*. Naturally or experimentally positive tissues provide the most representative sample for tissue validation studies. In some cases "spiked" samples have been demonstrated as not suitable samples for the validation of PCR to detect bacterial agents (Niedenhauser *et al*. 1993).

***Repeatability*** and ***Robustness*** refers to the ability of the assay to produce a consistent accurate result when repeated over a range of samples and under different laboratory conditions. To demonstrate repeatability and robustness of the PCR assay, tissues with varying concentrations of *S. agalactiae* are required to be tested by different operators in different laboratories.

**Experiment 1. Specificity of the PCR assay to detect *S. agalactiae***

**Bacterial strains and growth conditions**

Table 3 displays the identity and source of bacterial isolates used to validate the specificity of the assay. A collection of 22 *S. agalactiae* and 26 non-*S. agalactiae* bacterial isolates was retrieved from aquatic diagnostic samples from TAAHL collections (held at -80°C or in the freeze dried library) and cultured. Cultures were incubated on sheep blood agar (SBA) (Oxoid) for 48 hrs at 25°C. From pure culture, a single colony forming unit (cfu) from each strain was inoculated to 3 ml serum broth (SB) and incubated for 72 hours at 25°C.

**Table 3.** Bacterial strains used to validate the specificity of the *S. agalactiae* PCR assay

| Laboratory culture no. | Host | Bacteria - species | Year | Geographic isolation |
|---|---|---|---|---|
| *Streptococcus agalactiae* cultures | | | | |
| 8-43630 | Grouper | *Streptococcus agalactiae* | 2008 | Cairns |
| 8-43630 | Grouper | *Streptococcus agalactiae* | 2008 | Cairns |
| 9-60791 | Javelin Grunter | *Streptococcus agalactiae* | 2009 | Cairns |
| 9-62221 | Catfish | *Streptococcus agalactiae* | 2009 | Cairns |
| 8-43676 | Grouper | *Streptococcus agalactiae* | 2008 | Cairns |
| 8-43943 | Grouper | *Streptococcus agalactiae* | 2008 | Cairns |
| 9-61411 | Mullet | *Streptococcus agalactiae* | 2009 | Cairns |
| 9-63104 | Eastern Shovel nose Ray | *Streptococcus agalactiae* | 2009 | Gold Coast |
| 9-63104 | Mangrove Whiptail Ray | *Streptococcus agalactiae* | 2009 | Gold Coast |
| 9-64185 | Estuary Ray | *Streptococcus agalactiae* | 2009 | Gold Coast |
| 10-63890 | Human | *Streptococcus agalactiae* | 2010 | Townsville |
| 10-64220 | Crocodile | *Streptococcus agalactiae* | 2010 | Darwin |
| 10-64491 | Crocodile | *Streptococcus agalactiae* | 2010 | Darwin |
| 9-64491 | Estuary Ray | *Streptococcus agalactiae* | 2009 | Gold Coast |
| 10-61786 | Grouper | *Streptococcus agalactiae* | 2010 | Townsville |
| 10-62171 | Grouper | *Streptococcus agalactiae* | 2010 | Townsville |
| P10-71173 | Ray | *Streptococcus agalactiae* | 2010 | Gold Coast |
| P10-71173 | Ray | *Streptococcus agalactiae* | 2010 | Gold Coast |
| P10-71173 | Ray | *Streptococcus agalactiae* | 2010 | Gold Coast |
| P10-72452 | Queensland Grouper | *Streptococcus agalactiae* | 2010 | Gulf of Carpentaria |
| B09/1031 | Bovine Milk | *Streptococcus agalactiae* | 2009 | Gatton Vet School |
| B05-317 | Bovine Milk | *Streptococcus agalactiae* | 2009 | Gatton Vet School |
| Non- *Streptococcus agalactiae* cultures | | | | |
| 6-40280 | Barramundi | *Streptococcus iniae* | 2006 | Kelso, Townsville |
| 8-45981 | Sea Snake | *Steptococcus salivarius* | 2008 | Townsville |
| 5-42882 | Fish | *Streptococcus iniae* | 2005 | Darwin |
| 6-40593 | Barramundi | *Streptococcus iniae* | 2006 | Bowen |
| 8-46802 | Barramundi | *Streptococcus iniae* | 2008 | Mourlilyan |
| 8-47813 | Barramundi | *Streptococcus iniae* | 2008 | Kings Park |
| 2-42085 | Barramundi | *Flexibacter sp.* | 2002 | Gordonvale, North Queensland |
| 94-50115 | Tiger prawn | *Alteromonas sp.* | 1994 | Mossman, North Queensland |
| 4-41855 | Barramundi | *Vibrio harveyi* | 2004 | Bowen |
| 4-45482 | Barramundi Cod | *Vibrio splendidus 1* | 2004 | Bowen |
| 4-45981 | Banana prawn | *Vibrio alginolyticus* | 2004 | Cardwell |
| 4-46640 | Fish | *Shewanella putrefacians* | 2004 | Townsville |
| 4-47121 | Barramundi | *Aeromonas sobria* | 2004 | Lake Eacham, North Queensland |
| 4-46914 | Barramundi | *Vibrio splendidus 2* | 2004 | Bowen |
| 4-52250 | Barramundi Cod | *Vibrio sp.* | 2004 | Bowen |
| 4-56592 | Barramundi | *Vibrio mimicus* | 2004 | Wonga Beach, North Queensland |
| 5-40651 | Goldfish | *Aeromonas hydrophilia* | 2005 | Quality Control Unknown |
| 5-42576 | Barramundi | *Pseudomonas floursecens* | 2005 | Quality Control - Unknown |

| | | | | |
|---|---|---|---|---|
| 5-44583 | Barramundi | *Flavobacterium columnare* | 2005 | Townsville |
| 6-42495 | Barramundi | *Vibrio trachuri* | 2006 | Bowen |
| 6-46052 | Barramundi | *Pseudomonas stutzeri* | 2006 | Bowen |
| 6-49642 | Barramundi | *Vibrio damsela* | 2006 | Bowen |
| 4-46914 | Barramundi | *Vibrio harveyi* | 2004 | Bowen |
| 4-52072 | Barramundi | *Flexibacter sp.* | 2004 | Bowen |
| 10-61086 | White Spotted Grouper | *Vibrio proteolyticus* | 2010 | Cairns |
| 9-66443 | White Spotted Grouper | *Vibrio damsela* | 2009 | Cairns |

***DNA Extraction from bacterial broth cultures***

Three ml of serum broth cultures were centrifuged at ~3000 g for 30 min, to pellet the bacterial cells. The pelleted cells were re-suspended in 400 µL phosphate buffered saline (PBS) and subject to spin column DNA extraction using the Roche PCR template kit (Roche Pty. Ltd Cat. 11796828001), according to the manufacturer's instructions. DNA was eluted into 200 µL elution buffer and subject to PCR analysis.

***Polymerase Chain Reaction (PCR) assay***

To confirm the presence of *S. agalactiae,* PCR analysis was conducted on DNA template prepared from each of the broth cultures as previously described. The 25 µL PCR reaction consisted of: 5.0 µL 5X Coloured Buffer ($Mg^{2+}$ free), 0.75 µL $MgCl_2$ (50mM), 0.5 µL dNTP (10mM), 0.5 µL of each primer AgaF/AdyR (0.2 pmoles $\mu L^{-1}$), 0.5 µL of Mango Taq DNA polymerase (5U $\mu L^{-1}$ ) (Bioline), 1 µL of template and 19.2 µL RNAse free water. PCR amplification conditions were: 3 min at 94°C; followed by 40 cycles of 15 s at 94°C, 1 min at 58°C,30 s at 72°C; and a final extension of 5 min at 72°C. Reaction mixtures were held at 4°C until analysed by electrophoresis on a 1.5% agarose gel. A positive result was recorded if the expected amplicon size of ~845 bp was visualised.

**Experiment 2. Sensitivity of the PCR assay to detect *S. agalactiae***

***Culture and Serial dilution of Streptococcus agalactiae***

A *Streptococcus agalactiae (P10-72452)* culture was grown on SBA as described in Experiment 1. A colony was transferred to 3ml of serum broth and incubated for 48 h at 25 C. Following incubation, 100 µL of the suspension was transferred to 1 mL of 0.85% Saline. This process was repeated ten times resulting in ten, 10-fold serial dilutions of the culture. 100 µL of each dilution was then plated onto two SBA plates and incubated for 72 hours at 25 ºC.

***Plate count of serial dilutions***

After a 72 h incubation period, colonies were present on all plates up to a dilution of $10^8$. The number of colonies was recorded from duplicate plates of dilutions $10^6$ to $10^8$ which were the only plates which allowed resolution of individual colonies. The average number of the duplicate counts, for each plate of the $10^7$, were multiplied by ten, to represent the cfu/ ml.

***Sensitivity of PCR assay from serial dilution counts.***

The remaining 900 µL of each saline suspension were submitted to DNA extraction and PCR analysis as described in Experiment 1. Briefly the suspension was subjected to DNA extraction as described by the manufacturer's instructions as outlined above. No initial pelleting of the bacterial cells was required, as the total volume of 900 µL was suitable per the manufacturer's instructions. The 200 ul DNA extract was submitted for PCR analysis using methodology described previously. As the main scope of application of this assay is to

detect *S. agalactiae* in fish tissues it was considered most appropriate to express the sensitivity as cfu/ml. Sensitivity of the assay was extrapolated considering the concentration and dilution factors associated with extraction, compared to the calculated cfu/ml for culture plates from 2.1.

**Experiment 3: Robustness of the PCR assay.**
Robustness of the PCR assay was demonstrated by inter-laboratory testing of a panel of *S. agalactiae* positive and negative DNA extracts. A panel of 10 samples were each sent to The Berrimah Veterinary Laboratory (Northern Territory Department of Resources, Darwin) and Fish Health Laboratory (Western Australian Department of Agriculture, Perth) for PCR analysis using the methods outlined in experiment 1. DNA extracts, working instructions and all reagents were supplied to both co-operating laboratories and were identical to those outlined previously. Table 4 outlines details of the DNA extracts sent to the co-operating laboratories.

**Table 4.** Details and identifier of samples sent for Inter-laboratory demonstration of Robustness of the PCR Assay.

| **Identity of sample** | **Fish Health Laboratory (Western Australia) Sample No.** | **Berrimah Laboratory (Northern Territory) Sample No.** |
|---|---|---|
| 8-43630 *Streptococcus agalactiae* | 7 | 1 |
| 10-63890 *Streptococcus agalactiae* | 8 | 2 |
| 10-72452 *Streptococcus agalactiae 1:100 dilution* | 2 | 8 |
| 8-43943 *Streptococcus agalactiae* | 4 | 4 |
| 10-64220 *Streptococcus agalactiae* | 3 | 5 |
| 10-72452 *Streptococcus agalactiae no dilution* | 6 | 3 |
| 6-42495 *Vibrio trachuri* | 1 | 7 |
| 11-70749 *Streptococcus agalactiae* | 5 | 8 |
| P11-71289 *Streptococcus agalactiae** | Positive Control | Positive Control |
| 8-43640 *Streptococcus iniae** | Negative Control | Negative Control |

*P11-71289 and 11-70749 were *S. agalactiae* isolates obtained from the experimental challenge injection trial, labelled as Positive or Negative Controls.

**Experiment 4: Repeatability of the PCR assay.**
Eight DNA templates prepared from *S. agalactiae* and a negative control (6-42495) were tested by PCR according to the method already outlined. During an 8-week period the samples were tested in duplicate at TAAHL. Additionally four of the DNA templates were included as samples in the Inter-laboratory testing rounds to demonstrate robustness of the PCR assay. Details of the DNA template are provided in Table 5. Repeatability was initially described to involve 10 strains repeatedly tested over 8 weeks.

**Table 5.** Identity of *S. agalactiae* strains that underwent replicated testing

| **Identity of sample** | **Included in Inter-Laboratory Robustness** |
|---|---|
| 10-63890 *Streptococcus agalactiae* | Yes |
| 11-70749 *Streptococcus agalactiae* | Yes |
| 10-64220 *Streptococcus agalactiae* | Yes |
| 8-43630 *Streptococcus agalactiae* | Yes |
| 11-70746 *Streptococcus agalactiae* | No |
| 10-72452 *Streptococcus agalactiae* | No |
| B09-1031 *Streptococcus agalactiae* | No |
| B05/0317 *Streptococcus agalactiae* | No |
| Negative: 6-42495 *Vibrio trachuri* | No |

### 6.2.2 Results and Discussion

**Specificity of the PCR assay to detect *S. agalactiae***
The PCR assay demonstrated 100% specificity on bacterial cultures included in this validation. All 22 strains of *S. agalactiae* were detected by the PCR method outlined with the expected amplicon size of 845 bp visualised. No amplicons were visualised in any non- *S. agalactiae* cultures.

**Sensitivity of the PCR assay to detect *S. agalactiae***
Averaged plate counts on the $10^7$ dilution plate were 8.65 colonies. PCR analysis of the serial dilutions resulted in positive detections to the $10^3$ dilution. The level of detection of the PCR equated to 3 to 4 copies. This sensitivity level is comparable to the lowest dilution used to experimentally challenge juvenile *E. lanceolatus* as reported in the experimental injection challenge trial. The sensitivity of the assay to detect *S. agalactiae* from fish tissues was determined in section 6.3.

**Experiment 3: Robustness of the PCR assay to detect *S. agalactiae***
PCR results for the panel of DNA extracts sent to the collaborating laboratories are outlined in Table 6 below. Briefly, all expected positive and negative results were achieved by each laboratory, demonstrating the robustness of the PCR assay.

**Table 6.** Results and Identifiers of Inter-laboratory Testing of DNA Extracts to demonstrate Robustness of the PCR Assay. *Samples were provided as 'Positive' & 'Negative' Controls (labelled as such)

| **Identity of sample** | **Expected Result** | **Fish Health Laboratory (Western Australia)**<br><br>**Sample No. & Result** | **Berrimah Laboratory (Northern Territory)**<br><br>**Sample No. & Result** |
|---|---|---|---|
| 8-43630 *Streptococcus agalactiae* | Positive | 7: Positive | 1 Positive |
| 10-63890 *Streptococcus agalactiae* | Positive | 8: Positive | 2 Positive |
| 10-72452 *Streptococcus agalactiae* (1:100 dilution) | Positive | 2: Positive | 8 Positive |
| 8-43943 *Streptococcus agalactiae* | Positive | 4: Positive | 4 Positive |
| 10-64220 *Streptococcus agalactiae* | Positive | 3: Positive | 5 Positive |
| 10-72452 *Streptococcus agalactiae* (no dilution) | Positive | 6: Positive | 3 Positive |
| 6-42495 *Vibrio trachuri* | Negative | 1: Negative | 7: Negative |
| 11-70749 *Streptococcus agalactiae* | Positive | 5: Positive | 8: Positive |
| P11-71289 *Streptococcus agalactiae** | Positive | Positive | Positive |
| 8-43640 *Streptococcus iniae** | Negative | Negative | Negative |

**Experiment 4: Repeatability of the PCR assay**
Results of repeat testing of the eight *S. agalactiae* strains are provided in Table 7. Consistent positive detection of *S. agalactiae* was achieved with zero false positives. The repeatability of the assay is further supported by the inter-laboratory results also reporting positive detection of *S. agalactiae* on the same strains presented in Table 7. Samples which were used in inter-laboratory robustness have reported a total of 4 consistently repeated results. This data set met the OIE and SCAHL's guidelines.

**Table 7.** Results of PCR analyses for repeat testing of eight *S. agalactiae* strains.

| Identity of sample | Round 1 Result | Round 2 Result | Also Inter-Laboratory Robustness |
|---|---|---|---|
| 10-63890 *Streptococcus agalactiae* | Positive | Positive | Yes |
| 11-70749 *Streptococcus agalactiae* | Positive | Positive | Yes |
| 10-64220 *Streptococcus agalactiae* | Positive | Positive | Yes |
| 8-43630 *Streptococcus agalactiae* | Positive | Positive | Yes |
| 11-70746 *Streptococcus agalactiae* | Positive | Positive | No |
| 10-72452 *Streptococcus agalactiae* | Positive | Positive | No |
| B09-1031 *Streptococcus agalactiae* | Positive | Positive | No |
| B05/0317 *Streptococcus agalactiae* | Positive | Positive | No |
| 6-42495 *Vibrio trachuri* | Negative | Negative | No |

## Conclusion

The PCR assay detected all *S. agalactiae* (n=22) cultures with no false positives detected from any non-*S. agalactiae* culture (n=26). Sensitivity of the assay was demonstrated to be approximately 3 to 4 copies. Repeatability was demonstrated by the testing of *S. agalactiae* cultures over a series of repeat tests (n= 8 x 2). Robustness of the assay was demonstrated by inter-laboratory PCR testing done at two State Government Aquatic Diagnostic Laboratories. Both laboratories successfully detected *S. agalactiae* in a panel of ten unknown samples using the PCR assay. The broad guidelines provided by the OIE and SCAHLS demonstrated the validity of the PCR assay, using AgaF/AdyR primers, to identify *S. agalactiae* from laboratory cultures.

## 6.3 PCR validation in *E. lanceolatus* fish tissue samples from injection and immersion challenge trials

Validation is recognised as an on-going process depending on the application of the assay and the availability of appropriate samples representative of the scope of application of the assay. This section describes further validation of the PCR assay to detect *S. agalactiae* in DNA extracts prepared from bacterial isolations and fish tissues.

***Specificity*** of the PCR assay to detect *S. agalactiae* on DNA extracts from fish tissues was demonstrated using tissues collected from the injection challenge of *E. lanceolatus* (refer to Section 6.6). The injection challenge trial (Section 6.6) reported 100% morbidity, marked histopathology indicative of infection, and re-isolation of *S. agalactiae* from injected fish. The PCR assay conducted on the fixed fish tissues displayed 100% concordance with the histopathology and bacterial isolations obtained from the experimentally injected juvenile Queensland grouper *E. lanceolatus*. The sensitivity and specificity of the PCR assay on tissues from inoculated fish, which constitute a '*High*' positive sample group, was equal to that demonstrated by histopathology and bacterial isolation from fresh tissue.

Application of the PCR assay to *immersion* challenge fish which represents '*High*', '*Medium*' and '*Low*' concentration sample groups, demonstrated both specificity and sensitivity. The PCR assay displayed 67% concordance with the bacterial isolation assays. 100% of all fish which were positive by bacterial isolation were also positive by PCR. However a further six fish that were negative by bacterial isolation, were positive by the PCR assay. This result was to be expected, as the bacterial isolation technique does not detect *S. agalactiae* which has been killed by the fish immune response. The PCR assay displayed 79% concordance with histopathology. Fifteen of eighteen fish which were positive by histopathology were positive for detection of *S. agalactiae* by the PCR assay (Appendix 2). However *S. agalactiae* was detected by PCR in an additional ten fish which were determined negative or undetermined by histopathology. Concordance between the PCR and histopathology (75%) and PCR and bacterial isolation (66%) is comparable to that observed between the two "gold standard" techniques (77%).

The variable agreement between the PCR assay, bacterial isolation and histopathology was likely a result of differing sensitivities of the assays to detect *S. agalactiae*. The PCR demonstrated *Specificity* on all negative samples however was variably positive with samples from various exposure regimes.

Confirmation of the PCR assay results was planned to be done, using the FIHC assay developed at UQ (Section 6.4). The use of the FIHC assay on fish tissue samples would have provided further validation of results, allowing sensitivity, robustness and repeatability studies to be completed on fish tissues. Unfortunately, due to closure of the TAAHL by the Queensland Government, and resultant loss of staff and resources, this work was not able to be done.

### 6.3.1 Methods

**Experiment 1: Specificity of the PCR Assay to detect *S. agalactiae* on DNA Extracts from Fish Tissues.**

***Collection of tissues positive for S. agalactiae***

Section 6.6 reports the experimental injection challenge trial of juvenile *E. lanceolatus* with *S. agalactiae*. Fish were challenged by immersion or by injection. All fish from the injection groups ('*High*', '*Medium*' and '*Low*') displayed 100% morbidity and were confirmed to be positive for *S. agalactiae* by bacterial isolation, PCR analysis of the bacterial isolates and histopathology. Specificity was investigated in this validation study through optimising and applying the PCR assay to tissues collected from juvenile Queensland grouper *E. lanceolatus* that were experimentally injected with *S. agalactiae*. Samples of brain, head kidney and spleen of every fish in the experimental challenges were placed in 100% ethanol and shipped to TAAHL for PCR.

***Extraction of DNA from fish tissues***

DNA was extracted from fixed fish tissue using a commercial DNA extraction kit (Roche High Pure PCR template Kit Cat 11796828001). Briefly, tissues were removed from ethanol fixative and an approximately ~ 500mg tissue portion placed in a 1.7ml centrifuge tube (Fisher Biotech MCT-175-C) and stored overnight at 4ºC in 500ul phosphate buffered saline (PBS). The tissue was homogenised by hand-grinding using DNAase/RNAase free 1.5ml disposable pestles (Fisher Biotech. Cat.PES-15-B-SI). DNA extraction was conducted on the tissue homogenate according to the manufacturer's instructions. DNA template was eluted in 200µL Elution Buffer and subject to PCR analysis.

***Optimisation of the Polymerase chain reaction (PCR) for S. agalactiae detection using AgaF/AdyR***

Optimisation of the assay to detect *S. agalactiae* on fish tissues was required to achieve a level of sensitivity comparable to bacterial isolation and histopathology. Optimisation was conducted on DNA templates obtained from the '*High*' dose inoculation challenge groups and resulted in an increase in template and primer volume to the PCR reaction. The optimised assay for DNA template prepared from fish tissue homogenates consisted of 25 µL PCR reaction of: 5.0 µL 5X Coloured Buffer ($Mg^{2+}$ free), 0.75 µL $MgCl_2$ (50mM), 0.5 µL dNTP (10mM), 1.0 µL of each primer AgaF/AdyR (2 pmoles $\mu L^{-1}$), 0.5 µL of MangoTaq DNA polymerase (5U $\mu L^{-1}$) (Bioline), 5 µL of template and 11.25 µL RNAse free water. PCR amplification conditions were: 3 min at 94°C; followed by 40 cycles of 15 s at 94°C, 1 min at 58°C,30 s at 72°C; and a final extension of 5 min at 72°C. Reaction mixtures were held at 4°C until analysed by electrophoresis on a 1.5% agarose gel. A positive result was recorded if the expected amplicon size of ~845 bp was visualised.

***PCR analysis of confirmed positive fish tissues***

Following optimisation, PCR analysis to detect *S. agalactiae* was conducted on DNA templates from all fish tissues (as described above).

**Experiment 2: Sensitivity of the PCR assay to detect *S. agalactiae* on DNA Extracts from Fish Tissues**

Sensitivity was investigated in this validation study through applying the optimised PCR assay to tissues collected from juvenile Queensland grouper *E. lanceolatus* which were challenged by experimental immersion in *S. agalactiae*. Results from this experimental challenge trial suggest that samples from the immersion challenged groups contain varying concentrations of *S. agalactiae*.

***Collection of tissues positive for S. agalactiae in varied concentrations***.

Samples of brain, head kidney and spleen from every fish in the experimental immersion challenge groups (from the '*High*', '*Medium*' and '*Low*' dose treatment groups) were placed in 100% ethanol and transported to TAAHL for PCR analysis.

***Extraction of DNA from fish tissues***

Fish tissues were subject to DNA extraction as previously described.

***PCR analysis of confirmed positive fish tissues***

PCR analysis to detect *S. agalactiae* was conducted on DNA templates from all fish tissues using the optimised procedure as previously described.

### 6.3.2. Results and Discussion

**Experiment 1: Diagnostic Specificity of the PCR Assay to detect *S. agalactiae* on fish tissue homogenates.**

A summary of the PCR results on each fish from the injection challenge trial, with corresponding bacterial isolation and histopathology results, are presented in Table 8. Detailed PCR results from individual tissues from each fish are shown in Appendices 3 & 4. Concordance tables comparing PCR results to histopathology and bacterial isolation are presented as Tables 9 and 10 respectively. *S. agalactiae* was detected by PCR in 100% of the fish tissues which were positive by histopathology and bacterial isolation.

All tissues (brain, head kidney and spleen) from fish that were injected with *S. agalactiae* recorded a positive result by the PCR assay, regardless of exposure dose (n = 66 fish). All tissues (brain, head kidney and spleen) from the negative control group were negative by the PCR assay for the detection of *S. agalactiae* (n = 4 fish; Appendices 3 & 4). The PCR assay results from injected fish were supported by fish mortality and displayed 100% concordance with histopathology observed and bacterial isolation and PCR analysis of the isolates from isolations on fresh tissue reported in Section 6.6. Although fish were injected at varying concentrations mortality, bacterial isolation and histopathology suggested bacterial replication occurred in the fish and tissues obtained from the injected route represent samples which would considered '*high*' concentration positives. Thus, the result confirmed the specificity and sensitivity of the PCR assay on fixed fish tissues which would be considered 'high level' positive with respect to a validation sample.

**Table 8.** Summary of detection of *S. agalactiae* in fish tissues by PCR, histopathology and bacterial isolation from fish challenged with *S. agalactiae* by injection.
*R, Replicate 1; #R2, Replicate 2; +, positive; -, negative

| Treatments | PCR detection of *S.agalactiae* (TAAHL) (no. fish positive/total no. fish tested) | | Histopathology from fish tissue typical of GBS infection | Isolation of *S. agalactiae* from brain, kidney, liver, spleen on SBA (no. fish positive/total no. fish tested) | | PCR confirmation of *S. agalactiae* from bacteria isolated from brain, kidney, liver, spleen (no. fish positive/total no. fish tested) | |
|---|---|---|---|---|---|---|---|
| Treatment dose cfu $ml^{-1}$ (no. fish/treatment) | R1* | R2# | R1/R2 | R1 | R2 | R1 | R2 |
| $10^3$ (n=8) | + (6/6) | + (5/5) | + (11/11) | + (4/4) | + (2/4) | + (2/2) | + (2/2) |
| $10^4$ (n=8) | + (5/5) | + (4/4) | + (9/9) | + (4/4) | + (4/4) | + (2/2) | + (2/2) |
| $10^5$ (n=8) | + (5/5) | + (5/5) | + (10/10) | + (4/4) | + (4/4) | + (2/2) | + (2/2) |
| $10^6$ (n=8) | + (5/5) | + (5/5) | + (10/10) | + (4/4) | + (4/4) | + (2/2) | + (2/2) |
| $10^7$ (n=8) | + (6/6) | + (6/6) | + 12/12) | + (4/4) | + (4/4) | + (2/2) | + (2/2) |
| $10^8$ (n=8) | + (7/7) | + (7/7) | + (14/14) | + (4/4) | + (4/4) | + (2/2) | + (2/2) |
| Negative Control (n=8) | -( 8/8) | - (8/8) | - (8/8) | - (4/4) | - (4/4) | - (2/2) | - (2/2) |

**Table 9.** Comparison of the results of the PCR assay with bacterial isolation on inoculation challenged juvenile Queensland grouper *E. lanceolatus* (results per fish).

| | | Bacterial Isolation | |
|---|---|---|---|
| | | + | - |
| PCR Assay | + | 22 | 0 |
| | - | 0 | 4 |
| Agreement | | 26/26 | 100% |

**Table 10.** Comparison of the results of the PCR assay with histopathology on inoculation challenged juvenile Queensland grouper *E. lanceolatus* (results per fish).

| | | Histopathology | |
|---|---|---|---|
| | | + | - |
| PCR Assay | + | 66 | 0 |
| | - | 0 | 4 |
| Agreement | | 70/70 | 100% |

***Experiment 2: Diagnostic Sensitivity of the PCR Assay to detect S. agalactiae on fish tissue homogenates.***

A summary of the PCR results on each fish from the immersion challenge groups, along with corresponding bacterial isolation and histopathology results from Section 6.6 are presented in Table 11. Detailed PCR results from individual tissues from each fish are provided in Appendices 5 & 6.

Concordance tables comparing the results of the three assays are presented as Tables 12, 13 & 14 respectively. Concordance between the bacterial isolation and histopathology was 78% (Table 14). All negative control fish were negative by bacterial isolation and histopathology. Variation was observed between the “gold standard” results in all other treatment groups.

The PCR assay displayed varying concordance with the bacterial isolation and the histopathology results in all treatment groups except the negative controls. Briefly, *S. agalactiae* was detected by PCR in 100% of the fish which were positive for *S. agalactiae* by bacterial isolation. However the PCR detected *S. agalactiae* in a further six fish which were reported negative by bacterial isolation. All fish from the negative control group were negative by the PCR assay. Concordance between the PCR assay and bacterial isolation was 67% (Table 12).

The PCR assay displayed 75% concordance with the results of histopathology (Table 13). Fifteen fish which were positive by histopathology were also positive by the PCR assay for the detection of *S. agalactiae*. However, a further six fish were negative by histopathology yet positive by the PCR assay. In all of these cases, pathology was observed in the internal organs, but no bacteria were detected in internal organs examined histologically, or by Gram Glynn stains in five of six fish, nor were bacteria observed within tissues displaying pathology. In some cases, bacteria were observed in sloughed macrophages detected between gill filaments, however these cases were reported as negative by Gram Glynn special stains, since the bacteria were not present within the internal tissues of the fish, but had been shed by the fish in macrophages. No diagnosis was interpreted by histopathology in four fish. Twelve fish, including four negative controls, were negative by histopathology and the PCR assay. No fish that was positive by both bacterial isolation and histopathology, was negative for *S. agalactiae* by the PCR assay.

**Table 11.** Summary of detection of *S. agalactiae* in juvenile Queensland grouper tissue, by PCR, histopathology and bacterial isolation from fish challenged with *S. agalactiae* by immersion.
*R1=Replicate 1; R2=Replicate 2; + = positive; - = negative

| Treatments | PCR detection of *S.agalactiae* (TAAHL, DAFF) (No. fish positive/total number fish tested)) | | Histopathology from Fish Tissue typical of GBS infection | | Isolation of *S. agalactiae* from brain, kidney, liver, spleen on SBA (no. fish positive/total number fish tested) | | PCR confirmation of *S.agalactiae* on isolated collected from brain, kidney, liver, spleen (No. fish positive/total number fish tested) | |
|---|---|---|---|---|---|---|---|---|
| Treatment dose cfu fish$^{-1}$ (no. fish/treatment) | R1 | R2 | R1 | R2 | R1 | R2 | R1 | R2 |
| High Dose | + (2/4) | + (4/4) | +( 2/4) | +(4/4) | + (0/2) | + (2/2)* | N/a | + (2/2) |
| Medium Dose | + (2/4) | + (4/4) | +(1/4) | + (2/4) | + (0/2) | + (2/2) | N/a | + (2/2) |
| Low Dose | + (2/4) | + (3/4) | + (2/4) | + (1/4) | + (0/2) | + (0/2) | N/a | N/a |
| Positive Control (Injected) | + (4/4) | | + (4/4) | | + (2/2) | | + (2/2) | |
| Negative Control | (0/4) | | (0/4) | | (0/2) | | N/a | |
| Sentinel Control | +(0/4) | +(4/4) | +(0/4) | +(1/4) | (0/1) | (0/1) | N/a | N/a |

**Table 12.** Comparison of the results of the PCR assay and bacterial isolation on *immersion* challenged juvenile Queensland grouper *E. lanceolatus.*

| | | Bacterial Isolation + | Bacterial Isolation - |
|---|---|---|---|
| PCR Assay | + | 6 | 6 |
| | - | 0 | 6 |
| Agreement | | 12/18 | 67% |

**Table 13**. Comparison of the results of the PCR assay and histopathology on *immersion* challenged juvenile Queensland grouper *E. lanceolatus.*

| | | Histopathology* + | Histopathology* - |
|---|---|---|---|
| PCR Assay | + | 15 | 6 |
| | - | 3 | 12 |
| Agreement | | 27/36 | 75% |

*4 cases unresolved by histopathology (need confirmation by FIHC assay).

**Table 14**. Comparison of the results of histopathology and bacterial isolation on immersion challenged juvenile Queensland grouper *E. lanceolatus*.

| | | Histopathology* + | Histopathology* - |
|---|---|---|---|
| Bacterial Isolation | + | 6 | 0 |
| | - | 4 | 8 |
| Agreement | | 14/18 | 78% |

**Conclusion**

The PCR assay demonstrated high concordance with histopathology and bacterial isolation results to detect *S. agalactiae* from fish tissues. The variation in agreement between the assays is likely a result of varying levels of sensitivity. PCR has become a popular diagnostic assay due to its increased level of sensitivity in detecting the presence of an agent compared to other techniques.

Results of the PCR assay on the immersion challenge of juvenile Queensland grouper *E. lanceolatus* (section 6.6) suggest '*high*', '*medium*' and '*low*' level positive samples have been acquired, however as they are beyond the sensitivity of the bacterial isolation procedure, they cannot be definitively confirmed with present tools.

Histopathology is a subjective diagnostic tool which in this case may not suitable for identifying the presence of low or medium levels of *S. agalactiae.* Definitive confirmation of *S. agalactiae* (without the presence of a bacterial isolate) would require application of the *FIHC* assay that is specific for *S. agalactiae*.

Future application of the FIHC assay, to '*medium*' and '*low*' level positive samples, to further validate the assay on fish tissues, would have been useful to increase the validation beyond that provided in this section. The robustness and repeatability of the PCR assay for application to fish tissues, could be further strengthened, by future use of the FIHC by collaborating laboratories. Alternatively, the method of "spiking" negative tissues could be pursued.

Note that PCR analysis was conducted on over 1000 fish tissues from multiple fish species tested in Sections 6.8 & 6.9, with no "false" positive reactions detected by PCR, and only two spurious results resulting from post mortem degradation of samples.

## 6.4 Development of an immuno-histochemistry (IHC) method for detecting *S. agalactiae* in *E. lanceolatus* fish tissues

In order to aid detection and diagnosis of bacterial infection in juvenile grouper fish caused by infection with *S. agalactiae*, two specific detection methods, both used on histological fish tissue sections, were evaluated. The first method, fluorescence *in situ* hybridization (FISH), is based on detection of specific sequences of the 16S rRNA and immuno-labelling. This method uses antibodies to target specific antigens at the sub-cellular level on sections of biological tissue via specific epitopes. The FISH approach proved to be unsuitable for the detection of *S. agalactiae* on formalin-fixed and paraffin-embedded (FFPE) organs. Gram-positive bacteria such as *S. agalactiae* need an additional enzymatic treatment to open the thick peptide-glycan layer, in order to allow access of the probes to its target 16S rRNA, within the bacterial cells. Multiple attempts using various enzyme treatments and varying formamide stringency failed to give satisfactory results.

The alternative method, immune-histochemistry (IHC), was developed and optimised using a readily available commercial polyclonal antibody that reacts with type-specific carbohydrate on the surface of Group-B *Streptococcus* (GBS) such as *S. agalactiae.* Due to the high level of auto-fluorescence of fish tissues, when excited at wavelengths in the green, yellow and orange portion of the visible spectrum, detection of the primary antibody with minimum background was made possible using a commercial secondary antibody coupled with a photo stable far-red fluorescent dye. The FIHC approach for the detection and localization of GBS in fish tissues was specific and highly reproducible. Histological tissue sections from diseased and healthy *E. lanceolatus* were analysed using this method.

### 6.4.1. Methods

Both FISH and FIHC were performed on sections obtained from infected and healthy fish organs. Fish were infected via different routes: injection, immersion, cohabitation and oral (Sections 6.6 & 6.7), while healthy control animals were kept in a disease-free system. Briefly, tissues were fixed in 10% formalin for 48 h, paraffin embedded and sectioned (5µm).

**Fluorescence *in situ* hybridization**

For the FISH method, all oligonucleotide probes used in this study were previously described and evaluated (Kempf *et al*. 2000; Trabesius *et al*. 1998, 2000; Artz *et al*. 2013). Probes GBS 5’-GTAAACACCAAACMTCAGCG (Table 15) have been designed to target specifically the 16S rRNA region of *S. agalactiae*. The probes were synthetised and 5’-labeled with the cyanine dyes Cy5 and Cy3 (Sigma Aldrich, NSW, Australia). *In situ* hybridization was performed according to previous method (Amann *et al*. 1990). In brief, pure GBS bacterial cell suspensions and closely related streptococci (*S. iniae*, *S. pyogenes*) were immobilised by spotting an aliquot (20 µL) of fixed cell (PFA 4%) suspension on Teflon coated microscope slides (Biofusion Pty. Ltd.) and left to air dry at room temperature. Thereafter, cell membranes were permeabilised by an ascending ethanol series (50%, 80%, and 96%; 3 min each). The thick bacterial peptidoglycan layers were digested using lysozyme (5 mg $mL^{-1}$), mutanolysin (50 U $mL^{-1}$), pepsin (ready to use solution, Sigma), or proteinase K (100 µg $mL^{1}$) separately or in combination for 15, 30, 45 and 60 min. All enzymes were diluted in 10 mM Tris-HCl, pH 8.0. Enzymatic digestion was stopped with PBS and samples re-dehydrated in an ethanol series then air-dried. Twenty µL of hybridization buffer (0.9 M NaCl; 0.02 M TRIS-HCl pH 8.0; 0.01% SDS) containing 20% formamide and 25 ng of each

oligonucleotide was added and samples incubated overnight at 46°C in a humid chamber. After hybridization slides were transferred to a washing buffer (0.225 M NaCl, 0.02 M TRIS-HCl, 0.01% SDS) and incubated for 15 min at 48°C. Subsequently, DNA of both bacteria and host cells was stained with 1 $\mu g.mL^{-1}$ 4',6-diamidino-2-phenylindole (DAPI) for 5 min. GBS-specific probe labelled with the Cy3 or Cy5 dyes were applied simultaneously with probe Eub338-fluorescein isothiocyanate (FITC), complementary to a portion of 16S rRNA unique for the domain *Bacteria* (formerly known as *Eubacteria*). Aliquots of the same samples were tested in parallel with probe Non-Eub338, complementary to Eub338, to control non-specific binding probes.

Following tests on pure bacterial suspensions, the probes were used to examine histology slides from infected fish. Both whole bacterial cell preparations and FFPE tissues were analysed on the BX61 confocal laser microscopy platform (Olympus, Australia).

**Table 15**. Oligonucleotide probes used for FISH on whole bacterial suspension and FFPE fish tissues[a]

| Probe name | Sequences (5'-3') | Target | rRNA position[b] | Reference |
|---|---|---|---|---|
| GBS-Cy3[c] | GTA AAC ACC AAA CMT CAG CG | *S. agalactiae* | 16/67 | 1 |
| GBS-Cy5[d] | GTA AAC ACC AAA CMT CAG CG | *S. agalactiae* | 16/67 | 1 |
| Eub338-FITC[e] | GCT GCC TCC CGT AGG AGT | *Eubacteria* | 16/338 | 5 |
| NonEub338-Cy3 | ACT CCT ACG GGA GGC AGC | None[f] | None | 5 |

[a] A comparative analysis of almost 10,000 complete or almost-complete 16S rRNA sequences performed with the ARB software was used to develop oligonucleotide probes for FISH (Trebesius, Harmsen et al. 1998)
[b] First number, type of rRNA (16S); second number, target sequence position of first base of each oligonucleotide.
[c] Cy3 fluoresces in the yellow-green region (~550/570nm)
[d] Cy5 is fluorescent in the orange-red region (~650/670nm)
[f] Probe Non-EUB338, complementary to EUB338 was used to detect nonspecific binding of the oligonucleotides to microorganisms.

### Immuno-cytochemistry (ICC) & Immuno-histochemistry (IHC)

Alternatively to FISH, ICC was optimised on pure cultures of GBS and tested for specificity against other related streptococci (*S. iniae, S. pyogenes*), gram-positive (*Lactococcus lactis, Bacillus pumilus, Staphylococcus epidermidis*) and gram-negative bacteria (*Photobacterium damselae, Vibrio harveyi*), to be finally applied on FFPE fish tissue samples by IHC. Briefly, whole bacterial cell (WBC) suspensions were fixed in 4% PFA for 1h, rinsed in PBS and 15 µl of each suspension was immobilized onto coverslip (15 mm x 15 mm) coated with gelatin to 0.5% (v/v) and chromium potassium sulfate to 0.02% (w/v) for 10 min before being rinsed in distilled water, mounted and examined. For histological sections (5 µm) of FFPE organs, de-paraffinization was performed by dipping slides successively in xylene and ethanol 100% for 10 min twice each, then in ethanol 85%, 70% and distilled water for 5 min each. Both WBC and FFPE were permeabilised in 1X PBS/0.3% Triton X-100 (PBST) and non-specific binding sites were blocked using 10% goat serum in PBST for 3 h at 25°C. Samples were then incubated with a commercial primary rabbit polyclonal IgG antibody (Abcam, ab53584) diluted 1:400 in PBST and incubated overnight at 4°C. Primary antibody reacts with type-specific carbohydrate on the surface of GBS. Thereafter, samples were washed six times in PBS for 5 min to remove excess antibodies. Due to high level of auto-fluorescence of fish tissues (Figure 2), when excited at wavelengths in the green, yellow and orange portion of the visible spectrum, detection of the primary antibody with minimum background was made possible using a commercial (Abcam, ab150083) goat secondary antibody anti-rabbit IgG (1:400 in PBST) coupled with a photostable far-red fluorescent dye

Alexa Fluor® 647 for 2 h at room temperature. Tissues were counterstained with DAPI (1:2500) for 10 min and rinsed thoroughly in PBS before being mounted with an antifading polyvinyl alcohol mounting medium with DABCO® to retard photobleaching of fluorescent dyes. Coverslips were sealed with nail polish and allowed to cure overnight on a flat surface in the dark at 4°C before being imaged by microscopy.

**Imaging**

Entire slide tissue sections were scanned using an upright epifluorescent microscope Axio imager Z2 (Zeiss) equipped with a fully motorised X-Y-Z stage/automated robotic arm for loading slides from a feeder platform (Meta Systems). High-quality imaging automation was performed using a digital high-resolution progressive scan CCD camera (Cool Cube, Meta Systems). Slides were pre-scanned with a 5x (0.15) air objective (exposure time (et): 80ms; camera gain: 16.0). A focus map for each slide was created before slide was scanned with a 20x (0.8) air objective (camera gain: 7.0). Three-color images at each position were obtained using an ultra-fast colour LED illumination system (Meta systems) with the appropriate fluorescent filters: DAPI: nucleus in blue (et: 0.5 ms), Alexa 647: *S. agalactiae* in red (et: 33.3 ms) and Alexa 488: tissue autofluorescence in green used to contrast with other dyes and to visualize tissue structures (et: 7.8 ms). Tiled images were assembled with the V-slide software (Meta Systems) and saved as an imaris file (.ims).

### 6.4.2 Results and Discussion

**Fluorescence *in situ* hybridization: sensitivity & specificity**

In order to check if hybridization occurred correctly and for ease of visualisation of samples at higher magnification, bacterial suspensions were incubated with DAPI, which stained all bacterial DNA (Figure 2). Signal intensity obtained from hybridization with the Eub338-FITC probe on pure culture of GBS and *S. iniae* was quite low when compared with the DAPI micrographs (Figure 2). No signals were observed for *S. iniae* with the GBS-Cy3 probe confirming specificity of the probe to *S. agalactiae* (Figure 2). However, as for the Eub338-FITC probe, the level of hybridization with GBS-Cy3 on pure GBS culture was also very low suggesting low accessibility of the probes to their 16s rRNA targets regardless the enzymatic treatments used. Only a few positive bacteria were visible for Eub338 and GBS-Cy3 after a 12 h hybridization period at 46°C (Figure 2).

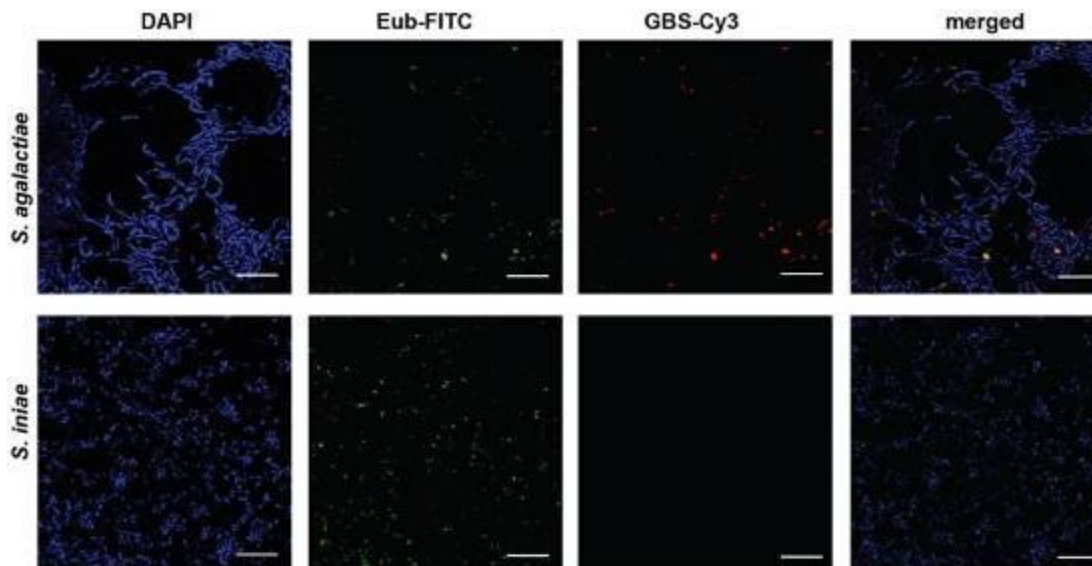


**Figure 2.** Fluorescence microscopy of microorganisms after FISH with various oligonucleotide probes. *S. agalactiae* (top row) and *S. iniae* (bottom row) were treated for 30 min with a enzymatic cocktail containing mutanolysin and proteinase K. Bacteria were stained with DAPI (blue signal; staining of DNA) following FISH with probe Eub338-FITC (green signal; specific for all *Eubacteria*), and probes GBS-Cy3 (red signal; specific for *S. agalactiae*). Magnification: 100X, scale bar = 10 µm.

**Autofluorescence**

Most cells and tissues have endogenous proteins and compounds that fluoresce naturally. It is an important consideration to keep in mind when imaging samples so there is no ambiguity between the autofluorescence of the sample with the signal of the marker of interest. Different tissues fluoresce in different ways, in the case of *E. lanceolatus* most organs are autofluorescent, an example is presented in Figure 3 with fish gills that fluoresce from the UV through to the red spectrum and can easily be confused with DAPI, Alexa488, and Texas-red labelling (Cy3) dyes. Autofluorescence decreased considerably in the far-red (Cy5) when the gills are excited at 647 nm (Figure 3.D). Therefore detection of GBS in fish tissues with minimum autofluorescence will require a dye whose excitation and emission spectrum is in the infrared.

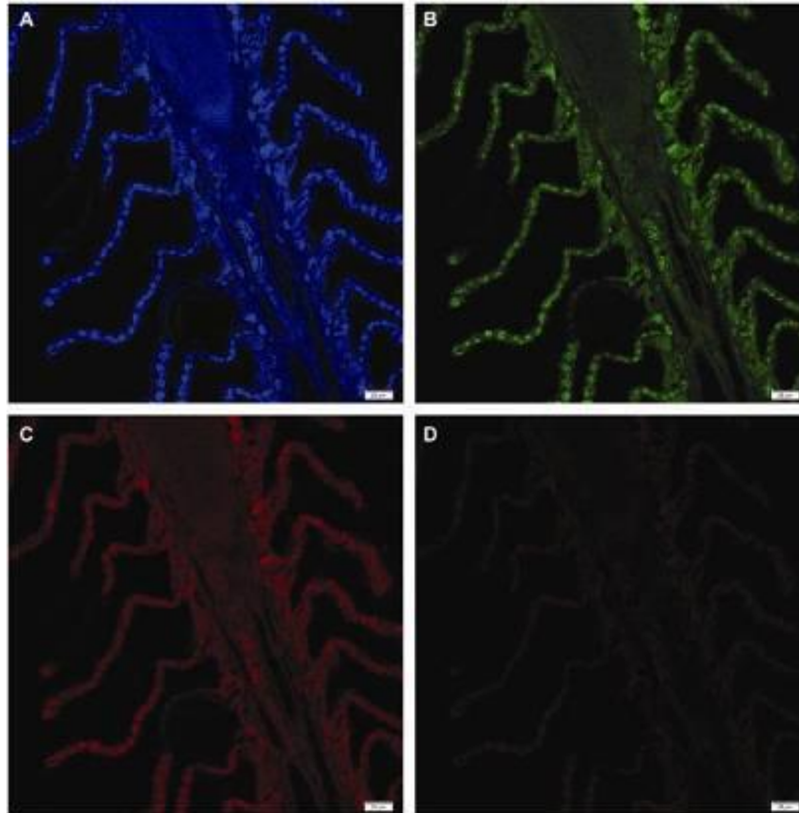


**Figure 3.** Autofluorescence of *E. lanceolatus* gills excited with different lasers on the BX61 Olympus confocal microscope. Probes and DAPI were omitted. Note the strong autofluorescence in the blue DAPI/UV 358 nm (A); in the green FITC/488 nm (B); in the red Cy3/550 nm (C) and the much lower autofluorescence in the far-red Cy5/IR 647 nm (D). Exposure time: 200 ms, magnification: 10X, scale bar = 10 μm.

**Conclusion of FISH**

Low accessibility of the probes to their target 16S rRNA, probably due to the complex polysaccharide capsule and highly impermeable peptidoglycan-dominated gram positive cell wall, made the FISH approach unsuitable for the detection of GBS in FFPE tissues. An alternative approach to this problem is to target specific epitopes (carbohydrates) that are readily present on the surface of the bacteria using specific antibodies, negating the need for the detection reagent to penetrate capsule and cell wall.

**Immuno-cytochemistry (ICC) validation on pure bacterial culture**

The specificity of the primary antibody was validated on pure bacterial suspensions by immuno-cytochemistry. All negative controls *S. iniae, S. pyogenes, L. lactis, B. pumilus, S. epidermidis, V. harveyi* and *P. damselae* were not recognized by the primary antibody anti-carbohydrates (Figure 3B). In contrast, GBS isolates were positive (Figure 3A). Bacteria present in the bright field micrographs of both GBS isolates tested were positive by fluorescence imaging appropriate to the AlexaFluor 647 excitation and emission wavelengths (Figure 3A). Once ICC was validated and optimized on pure culture, primary and secondary antibodies were used on FFPE tissues. Both new and archival histological sections from diseased and healthy Queensland groupers, *E. lanceolatus*, were analysed by immuno-histochemistry.

**Immuno-histochemistry on Queensland grouper tissues**

GBS was detected on FFPE tissues sectioned from organs originated from different challenge

trials (injection, immersion, oral and cohabitation) all performed previously (see Sections 6.6 & 6.7). For positive control, sections were obtained from fish injected i.p with a dose of $10^6$ cfu $fish^{-1}$ of GBS and organs from healthy animals kept in a system free of disease at the Northern Fisheries Centre (NFC) in Cairns were sectioned for negative controls.

Histopathological observations made by light microscopy on haematoxylin and eosin (H&E) and Gram-Glynn stains revealed colonies of typical coccoid bacteria, which were observed in most organs of infected animals with accompanying inflammation (Figure 5). IHC performed on sections of these same organs confirmed that these coccoid bacteria were indeed GBS (Figure 4).

Pathological analysis of fish infected with *S. agalactiae* displayed consistent pathology including ophthalmitis, exophthalmos, keratitis, meningitis, branchitis, splenitis, interstitial nephritis, hepatitis, gastritis, enteritis, pancreatitis, peritonitis, myositis and dermatitis with predominant lymphocytic granulomatous inflammatory response, consisting of aggregations of macrophages containing fluorescent coccoid bacteria. Control fish displayed some very minor pathology, but no GBS bacterial cells were detected by IHC in these samples.

**Conclusion**

Detection of Group-B *streptococcus* (GBS) in fish tissues by IHC was specific and highly reproducible. The localisation of the bacteria in the different organs that was revealed by immuno-fluorescence, corresponded with the Gram-glynn observations. IHC is a more informative method over routine H&E in that it can detect a pathogen in tissues in a very specific manner.

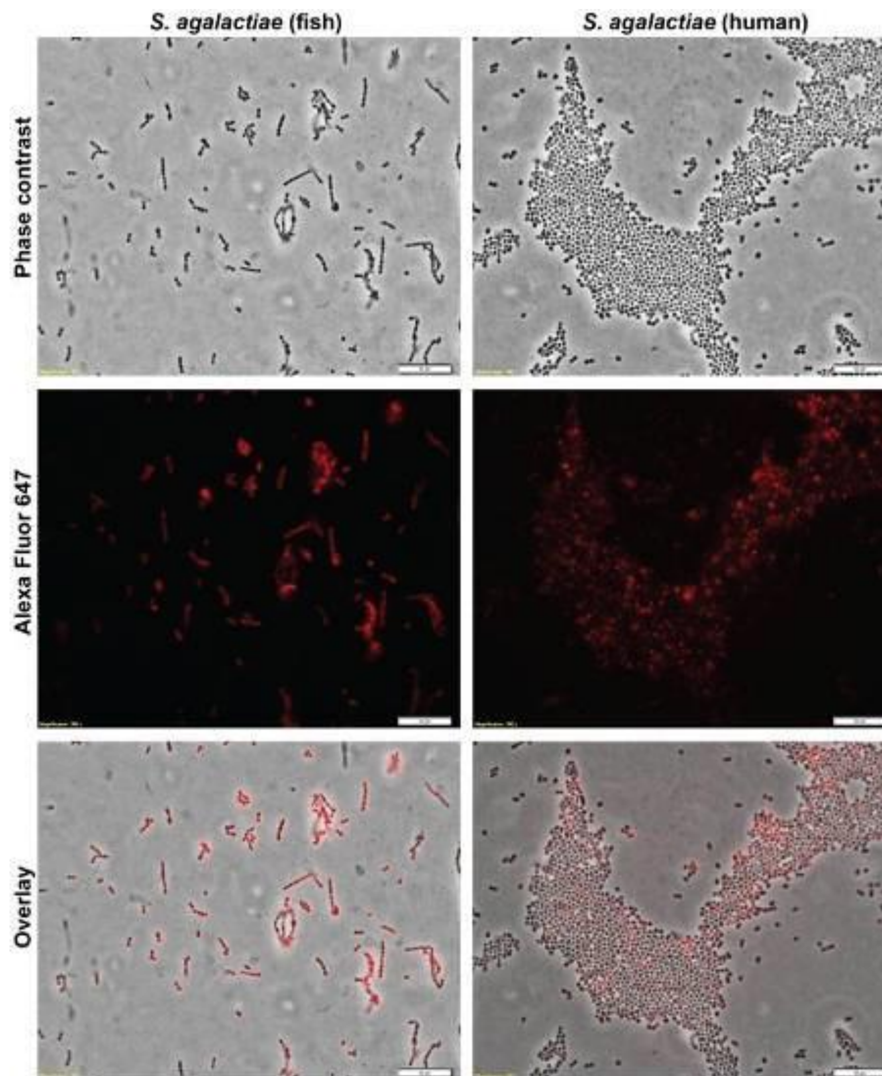


**Figure 4.** Fluorescence microscopy of microorganisms after immune-cytochemistry with primary antibody (ab53584), and secondary antibody (ab150083). *S. agalactiae* fish isolate QMA0281 (left) & human isolate QMA0370 (right). Phase contrast, Alexa Fluor 647 and overlay images of the former two were obtained for each bacterium. Both fish and human GBS isolates are positive for Alexa Fluor 647. Note how bacteria can be arranged either in chain or in individual coccus. Bar = 10 μm.

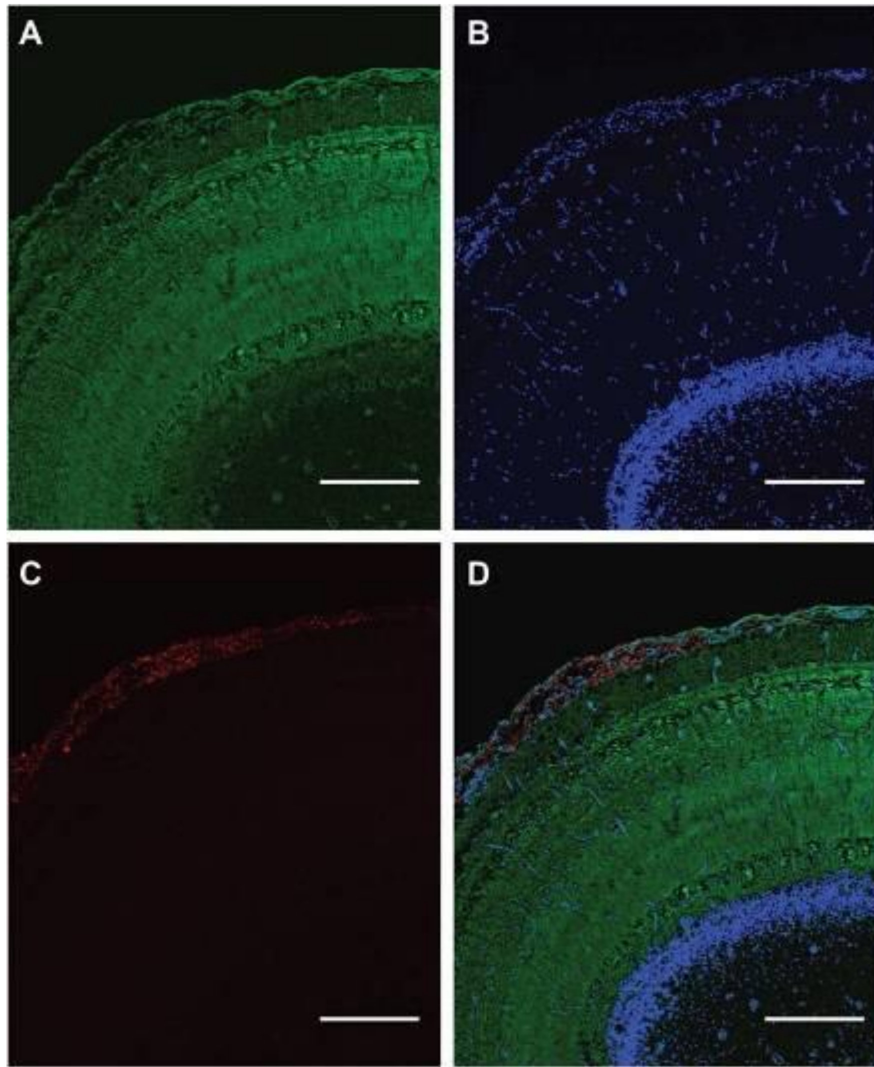


**Figure 5.** Photomicrographs of a lateral section of the optic tectum of the brain of *E. lanceolatus* injected i.p with *S. agalactiae*. (A) Green, autofluorescence of the tissue used as a counterstain in the FITC channel (B) Blue, nuclei DNA stained with DAPI. (C) Red, GBS bacteria positive in the Alexa Fluor 647 channel. (D) Overlay image of the three channels DAPI, FITC and Alexa Fluor 647. Magnification: 10X, scale bar = 100 µm.

## 6.5 Molecular typing of *S. agalactiae* isolates for epidemiological tracing

**Background**

Mortalities in wild Queensland Grouper (*E. lanceolatus*) resulting from infection by *S. agalactiae* (group B *Streptococcus*, GBS) were first recorded in 2008. Ninety-five individual adult *E. lanceolatus* were reported dead since the index case, although isolation of *S. agalactiae* and associated pathology was not confirmed in all 96 cases. In 2009, sick javelin grunters were sampled by QDAFF staff in Cairns, and subsequently analysed at TAAHL, were found to be infected by *S. agalactiae*. Following this finding, a small-scale survey was conducted by Biosecurity Queensland, TAAHL, and a mullet was found infected with *S. agalactiae*. *S. agalactiae* was also subsequently isolated from several species of wild stingrays, from two separate disease epizootics that occurred at *Sea World* (Bowater *et al*. 2012).

Establishing a possible origin and route of spread of *S. agalactiae* is important for several reasons, as outlined in the original FRDC research proposal. Firstly, this is the first recorded occurrence of *S. agalactiae* in wild fish in Australia, yet *S. agalactiae* has caused widespread mortalities in several cultured fish species and caused wild fish kills in many countries (Evans *et al*. 2002). Determining the source of the Australian outbreak is critical to develop biosecurity measures, to protect Australian aquaculture industries and wild fish stocks. Secondly, *S. agalactiae* is one of the major causes of meningitis in human neonates, and human pathogenic variants have been reported to infect fish (Evans *et al*. 2008), and establishing the genetic background of the grouper isolates is a matter of public safety and reassurance. Finally, an understanding of where the Australian strains of *S. agalactiae* are likely to have arisen is important, since this knowledge can assist and contribute to the development of state and national biosecurity policies, strategies and control measures to assist in protecting native fauna, preventing further infections from entering other wild fish populations in Australia, and identifying where interventions can be made to minimise biosecurity risks to Australian fisheries and aquaculture industries.

### 6.5.1 Methods

Two molecular methods were used for epidemiological tracing of *S. agalactiae*, namely multilocus sequence typing (MLST) and molecular serotyping. MLST was chosen as a useful method, since it does not require physical collection of bacterial reference strains from around the world, but permits accurate comparison with global databases of bacterial isolates online. Multilocus sequence typing (MLST) is based on short sequence reads (400-500bp) of 7-8 housekeeping genes within the core genome of many bacterial genera and species (Enright *et al*. 2001; Jones *et al*. 2003; Maiden *et al*. 1998). A public database was established more than ten years ago (pubMLST.org) that is very rich in sequence-typed *S. agalactiae* strains (Bentley *et al*. 2006; Jones *et al*. 2003; Luan *et al*. 2005), including many fish isolates (Evans *et al*. 2008). The second method used was molecular serotyping, which provides greater resolution at the local scale than MLST, and is also physiologically relevant as it explores the genes encoding the surface epitope determinants of the capsular polysaccharide (cps) (Honsa *et al*. 2008; Kong *et al*. 2008). In our original proposal we outlined using MLST-type and capsular-polysaccharide (*cps*) genotyping of Australian GBS isolates to establish origin and connectivity using Sanger sequencing. Subsequent to our proposal, next generation sequencing (NGS) technologies became more attractive as their availability and cost fell dramatically. More importantly, there has been a substantial advance in the software pipelines required for analysis of the data generated by these methods. Consequently we applied for,

and were granted, an amendment to the initial proposal to permit sequencing of the entire genomes of 23 *S. agalactiae* isolates from Australia by Illumina HiSeq NGS. This would enable delivery all of the information required for MLST and *cps* serotyping, but would also offer the potential to discover novel markers associated with Australian pathogenic isolates further down the track, and at no additional cost to the original proposal. The isolates have now been sequenced and the MLST and cps data are provided in this report.

***Bacterial strains***
Twenty-three *S. agalactiae* bacterial isolates (including one internal reference, COH-1) were selected for genotyping and are presented in Table 16.

***Genomic DNA extraction from bacterial broth culture***
High molecular weight genomic DNA (gDNA) was isolated from Group-B *Streptococcus* using a commercial kit (Genomic–tip 20/G, Qiagen) in accordance with the manufacturer's protocol. Resulting DNA was checked for quantity using a commercial fluorometric technology (QuBit, Invitrogen), which is based on specific molecular probes dyes to quantitate nucleic acid of interest. Once bound to the double-stranded molecule of DNA, these fluorescent dyes emit signals that can be measured. DNA quality was visualised by electrophoresis on a 1% agarose gel to check for absence of gDNA shearing before sequencing.

***Genome sequencing***
Genomic fragment libraries for whole genome sequencing were prepared at the Australian Genome Research Facility (AGRF) using Illumina TruSeq DNA library preparation protocol. Briefly, 1 µg DNA was fragmented using Covaris shearing followed by an end-repair (3'→5' exonuclease and polymerase fill in) to generate blunt ends. Fragments were A-tailed then TA-ligated onto Illumina adaptors. Ligated DNA fragments were size selected by gel electrophoresis and gel excision to create 23 libraries (1 per isolate) for subsequent sequencing. Libraries were pooled for sequencing on the Illumina HiSeq 2000 instrument at the AGRF according to manufacturer's protocols. Paired-end 100-bp sequence reads with an estimated insert size average of 370 bp were generated for the 24 isolates, including the previously sequenced strain COH1 (QMA0370) used as an internal reference. For each isolate, between 5,288,952 and 12,577,340 read pairs were obtained, corresponding to an average coverage of ~670X to ~1,400X. Base calling was processed with Illumina RTA software v1.10.36. De-multiplexing and conversion to FastQ format were performed with CASAVA v1.8.2. FastQC was used to assess the quality of the sequence reads and to look for the presence of contaminating adapters. When required, additional inspection using in-house tools were performed. Due to the excess coverage obtained in most samples, we chose random subsets of one million read pairs for each strain for subsequent analyses, which corresponds to an average coverage of 100X.

**Table 16.** List of the GBS isolates used in this project. Strains underlined have been isolated from different organs of the same animal. TAAHL= Tropical & Aquatic Animal Health Laboratory; GVS=Gatton Veterinary School; QUT=Queensland University of Technology; Anon=anonymous donor, hospital; Gulf=from the Gulf of Carpentaria, Queensland.

| Accession Number | Host Common name | Host Scientific name | Year of isola-tion | Geographi--cal isolation | Isolate provi-ded by | organ of isolation | [Stock] dsDNA | Units | Vol (µL) (TE) | Qua-ntity µg |
|---|---|---|---|---|---|---|---|---|---|---|
| QMA0284 | Grouper 1 | *Epinephelus lanceolatus* | 2008 | Cairns | TAAHL | Eye | 137 | ng/µL | 40 | 5.48 |
| QMA0285 | Grouper 2 | *Epinephelus lanceolatus* | 2008 | Cairns | TAAHL | Head kidney | 164 | ng/µL | 40 | 6.56 |
| QMA0267 | Grouper 3 | *Epinephelus lanceolatus* | 2008 | Cairns | TAAHL | Eye | 216 | ng/µL | 40 | 8.64 |
| QMA0280 | Grouper 4 | *Epinephelus lanceolatus* | 2010 | Townsville | TAAHL | Kidney | 424 | ng/µL | 20 | 8.48 |
| QMA0281 | Grouper 5 | *Epinephelus lanceolatus* | 2010 | Townsville | TAAHL | Heart | 147 | ng/µL | 30 | 4.41 |
| QMA0368 | Grouper 6 | *Epinephelus lanceolatus* | 2010 | Topsy creek, Gulf | TAAHL | Eye | 151 | ng/µL | 40 | 6.04 |
| QMA0369 | Grouper 7 | *Epinephelus lanceolatus* | 2011 | Cape Cleveland, Townsville | TAAHL | Eye | 122 | ng/µL | 40 | 4.88 |
| QMA0268 | Javelin grunter | *Pomadasys kaakan* | 2009 | Cairns | TAAHL | Caudal Kidney | 456 | ng/µL | 40 | 18.24 |
| QMA0287 | Javelin grunter | *Pomadasys kaakan* | 2009 | Cairns | TAAHL | Heart | 191 | ng/µL | 40 | 7.64 |
| QMA0271 | Catfish | *Arius thalassinus* | 2009 | Cairns | TAAHL | Heart | 374 | ng/µL | 40 | 14.96 |
| QMA0290 | Catfish | *Arius thalassinus* | 2009 | Cairns | TAAHL | Caudal kidney | 156 | ng/µL | 40 | 6.24 |
| QMA0274 | Mullet | *Liza vaigensis* | 2009 | Cairns | TAAHL | Heart | 436 | ng/µL | 40 | 17.44 |
| QMA0275 | Eastern shovelnose ray 1 | *Aptychotrema rostrata* | 2009 | *Sea World* (SE Qld) | TAAHL | Internal organs | 412 | ng/µL | 40 | 16.48 |
| QMA0276 | Mangrove whiptail ray 2 | *Himantura granulate* | 2009 | *Sea World* (SE Qld) | TAAHL | Internal organs | 408 | ng/µL | 30 | 12.24 |
| QMA0277 | Estuary ray 3 | *Dasyatis fluviorum* | 2009 | *Sea World* (SE Qld) | TAAHL | Internal organs | 370 | ng/µL | 30 | 11.1 |
| QMA0320 | Estuary ray 5 | *Dasyatis fluviorum* | 2010 | *Sea World* (SE Qld) | TAAHL | Brain | 516 | ng/µL | 40 | 20.64 |
| QMA0336 | Crocodile | *Crocodylus porosus* | 2010 | Darwin | TAAHL | Joint | 348 | ng/µL | 40 | 13.92 |
| QMA0370 | Human | *Homo sapiens* | 2006 | United States | QUT | infant septicae-mia | 73.8 | ng/µL | 15 | 1.107 |
| QMA0355 | Human | *Homo sapiens* | 2011 | Townsville | anon | Foot | 586 | ng/µL | 40 | 23.44 |
| QMA0357 | Human | *Homo sapiens* | 2011 | Townsville | anon | Female genital | 538 | ng/µL | 40 | 21.52 |
| QMA0306 | cow | *Bos taurus* | 2005 | unknown | GVS | Bovine milk | 588 | ng/µL | 40 | 23.52 |
| QMA0300 | dog | *Canis canis* | 2008 | unknown | GVS | Canine urine | 408 | ng/µL | 40 | 16.32 |
| QMA0303 | cat | *Felis felus* | 2009 | unknown | GVS | Feline left fore paw | 472 | ng/µL | 40 | 18.88 |

### *Assembly and preliminary comparative genome analysis*

Due to the high level of adapter contamination observed for a few samples, various assembly strategies were investigated to optimise assemblies and coverage yield. *De novo* assembly of reads obtained from each isolate was performed using Velvet 1.2.02, following preliminary filtering of sequence reads with a minimum quality of Q30 and selective trimming of low quality 3' ends down to 70 bp maximum. Preliminary automated annotation was then performed using Prokka. Comparison and mapping visualization was undertaken using a combination of the software Mauve, Artemis, BRIG and Easyfig (Sullivan *et al*. 2011).

***MLST***

The *S. agalactiae* MLST database (www.mlst.net) is a data repository containing typing sequence information of the following seven housekeeping gene fragments: alcohol dehydrogenase, *adhP*; phenylalanyl tRNA synthetase, *pheS*; glutamine transporter protein, *atr*; glutamine synthetase, *glnA*; serine dehydratase, *sdhA*; glucose kinase, *glcK*; and transketolase, *tkt*. Each different sequence at a locus gives a different allele number. The combination of these alleles gives the allelic profile of the isolate. The whole draft genome sequence of each isolate was submitted directly to the MLST database website where a BLAST search was performed to determine each individual allelic profile and sequence type. One novel allelic variation was discovered that required confirmation by PCR and Sanger sequencing as follows. To avoid any error from sequencing, two separates PCR reactions from independent DNA templates to amplify the *glnA* locus (QMA0368) were performed with the respective amplification primers (glnAAF/AAR) (Table 17). For sequencing, PCR products were cleaned with shrimp alkaline phosphatase/ exonuclease I and sequenced in both directions with the respective sequencing primers (glnASF/ASR) by AGRF (Table 17).

**Table 17**. Primers used to amplify and sequence the glnA MLST locus by Sanger sequencing.

| Locus | CMS Ref. | Locus (5' to 3') | Sequences | Tm (°C) | Amplicon size (bp) |
|---|---|---|---|---|---|
| ginA | UQ-GBS-ginAAF | ginA amplification F | CCGGCTACAGATGAACAATT | 61,5 | 589 |
| | UQ-GBS-ginAAR | ginA amplification R | CTGATAATTGCCATTCCACG | 62,8 | |
| | UQ-GBS-ginASF | ginA sequencing F | AATAAAGCAATGTTTGATGG | 56,6 | 498 |
| | UQ-GBS-ginASR | ginA sequencing R | GCATTGTTCCCTTCATTATC | 58,4 | |

***Molecular serotyping***

Serotyping of *S. agalactiae* provides higher discriminatory power for epidemiological tracing and is also immunologically relevant (Kong *et al*. 2008). Complete sequence of the capsular polysaccharide synthesis *cps* operon of each isolate was derived from the draft genome sequence. Reference sequences for the ten serotypes described so far in the literature (Ia, Ib, Ic, II to VIII) were retrieved from the GenBank database on the NCBI website (Table 18). BLASTn comparisons were then performed to determine the serotype of each isolate. Visualisation was performed using Easyfig on a set of isolates representative of the different serotypes characterized.

**Table 18**. Accession No. of the complete cps sequences of the reference strain for each existing serotype.

| Strain | Serotype | Accession No. |
|---|---|---|
| 515 | Ia | NZ_AAJP00000000.1 |
| | Ib | AB050723 |
| A909 | Ic | NC_007432 |
| | II | AY375362 |
| COH1 | III | AF163833 |
| NEM316 | III | NC_004368.1 |
| CNTC 1/82 | IV | AF355776 |
| 2603V/R | V | NC_004116.1 |
| CNTC 1/82 | V | AF349539 |
| NT6 | VI | AF337958 |
| 7271 | VII | AY376403 |
| SMU014 | VIII | AY375363 |

### 6.5.2 Results & Discussion

Illumina sequencing on 23 isolates (Table 16) has been completed and generated sufficient data to allow further analysis (Table 19). As it is often the case when sequencing small bacterial genomes with high throughput techniques, data yield was very good but resulted in an average coverage too high to be manageable for downstream analysis (~670X to ~1,400X). A good range for coverage is around 100-200X, thus reads for each isolate were sub-sampled down to 100X, to facilitate the assembly process, data handling and visualisation.

**Table 19**. Illumina number of 100bp paired end reads and data yield (bp) for each of the isolate. Flowcell ID: BD0HH8ACXX

| Lane | Sample Name | Paired Reads | Data Yield (bp) |
|---|---|---|---|
| 2 | QMA0265/Grouper 2 | 10,870,098 | 2.17 Gb |
| | QMA0267/Grouper 3 | 9,793,421 | 1.96 Gb |
| | QMA0268/Javelin grunter | 6,450,256 | 1.29 Gb |
| | QMA0271/Catfish | 5,817,315 | 1.16 Gb |
| | QMA0274/Mullet | 7,939,677 | 1.59 Gb |
| | QMA0275/E.S ray | 5,288,952 | 1.06 Gb |
| | QMA0276/MW ray | 7,088,617 | 1.42 Gb |
| | QMA0277/Estuary ray 1 | 6,333,588 | 1.27 Gb |
| | QMA0280/Grouper 4 | 6,036,741 | 1.21 Gb |
| | QMA0281/Grouper 5 | 11,279,969 | 2.26 Gb |
| | QMA0300/Dog | 9,615,983 | 1.92 Gb |
| | QMA0303/Cat | 8,376,926 | 1.68 Gb |
| 3 | QMA0306/Cow | 11,299,609 | 2.26 Gb |
| | QMA0320/Esturay Ray 5 | 8,869,615 | 1.77 Gb |
| | QMA0336/Crocodile | 10,083,887 | 2.02 Gb |
| | QMA0355/Human foot | 11,217,770 | 2.24 Gb |
| | QMA0357/Female genitale | 10,756,122 | 2.15 Gb |
| | QMA0368/Grouper 6 | 10,333,663 | 2.07 Gb |
| | QMA0369/Grouper 7 | 12,577,340 | 2.52 Gb |
| | QMA0284/Grouper 1 | 10,364,253 | 2.07 Gb |
| | QMA0285/Grouper 2 | 11,270,428 | 2.25 Gb |
| | QMA0287/Javelin grunter | 8,861,670 | 1.77 Gb |
| | QMA0290/Catfish | 12,616,502 | 2.52 Gb |
| | QMA0370/COH1/Inter REF | 8,794,149 | 1.76 Gb |
| **Total** | | **221,936,551** | **44.39 Gb** |

Assembly optimisation was hampered by the high level of adapter contamination of 8 samples out of the 23 sequenced. This was resolved by stringent data filtering (Q>30, end trimming, both reads of a pair discarded if one read fails the quality step). The internal reference COH1 is a draft genome (incomplete), so comparisons are limited as gaps are present in the original reference sequence. The complete genome of NEM316 (human isolate,

serotype III) was therefore used for comparison, although it has not been sequenced internally in this experiment.

In spite of these issues, all the quality controls were completed and parameters for optimal assembly confidence of the 23 GBS genomes have been established. Assembly is the most critical step as all downstream analyses rely on accuracy of the assembly. Results of the assembly indicate that the size of the marine genomes are smaller compared to those of human and terrestrial isolates, (1.8 Mb compared with 2.1-2.2 Mb), suggesting a reduction in size of the marine genomes of approximately 300 to 400 kb. While this needs to be investigated further, this is consistent with prior observations on other fish pathogens sequenced to date (Bisharat *et al*. 2007; Wiens *et al*. 2008).

***16S rRNA phylogeny***

In the context of a whole genome comparative study using a short read high throughput sequencing technique such as Illumina, 16S rRNA typing lacks sensitivity to determine accurately the genetic relatedness of all the isolates. This is mainly due to rRNA regions being present in several copies, which can often harbour microvariations between copies. Considering that whole genome information will be available for all isolates, a comprehensive phylogenetic analysis based on the core genome was performed instead.

***MLST***

Sequence Type (ST) for each of the isolate was determined using the BLAST search available on the MLST database website, in the section dedicated to *S. agalactiae*. Serotypes were determined using reference sequences (Table 20) for each of the 10 serotypes (Ia, Ib, Ic, II to VIII). Serotype, ST and other phenotypic properties of all Group B *Streptococcus* strains used in this study are presented in Table 20. Corresponding allelic profile of the seven housekeeping genes for each of the ST found in this study are summarized in Table 21.

All the Australian piscine isolates belong to the ST 261 lineage, a sequence type previously reported in a farmed Nile tilapia from Israel in 1994 (ATCC 51487). ST 261 is related to ST 257 (Nile tilapia, Brazil) and forms, along with the other more distant STs identified in that study (ST 258 (Hybrid striped bass, Israel), ST 259 (Nile tilapia, USA) and ST 260 (Nile tilapia, Honduras)), a phylogenetic group distinct from human and bovine isolates (Evans *et al*. 2008) (Figure 6). Although each ST was confined to a single geographical location, it is suggested that they belong to a population of GBS that has a broad worldwide distribution and predominantly causes disease in fish. Much less variability was observed in this study with all but one of the piscine isolates belonging to the same clone ST 261, despite being selected from various locations, fish species, infection sites and date range (2008 to 2011). As the ST 261 outbreak occurred more than 15 years ago in Israel, this new outbreak could suggest that ST 261 is a variant particularly successful in spreading and causing disease in a wide range of fish. Piscine GBS isolates from Australia appeared to represent a distinct genetic population of strains. The MLST confirmed the divergence of all piscine isolates from human, cow, crocodile, cat and dog isolates, as they do not share any allele with their respective STs (Figure 6).

**Table 20.** Piscine, crocodile, human, bovine, dog and cat GBS phenotypic properties.

VP, Voges-Proskauer reaction; HIP, hippuric acid; ESC, esculin ferric citrate; PYRA, pyrrolidonyl arylamidase; αGAL, α-galactosidase; βGUR, β-giucuronidase ; β-GAL, β-galactosidase; PAL, alkaline phosphatase; LAP, leucine aminopeptidase; ADH, arginine dihydrolase; RIB, ribose; ARA, arabinose; MAN, mannitol; SOR, sorbitol; LAC, lactose; TRE, trehalose; INU, inulin; RAF, raffinose; AMD, amidon; GLYG, glycogen; +, positive reaction; - negative reaction; NT, non-typable. Shaded areas denote differences between isolates.

| Isolates | Serotype | ST | CC | VP* | HIP* | ESC* | PYRA* | αGAL* | βGUR* | βGAL* | PAL* | LAP* | ADH† | RIB† | ARA | MAN† | SOR† | LAC† | TRE† | INU† | RAF† | AMD† | GLYG† | β HEM† |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Fish** | | | | | | | | | | | | | | | | | | | | | | | | |
| Grouper 1 (QMA0284) | Ib | 261 | | + | + | - | - | - | + | - | + | + | + | + | - | - | - | - | - | - | - | - | - | - |
| Grouper 2 (QMA0285) | Ib | 261 | | + | + | - | - | - | + | - | + | + | + | + | - | - | - | + | - | - | - | + | - | - |
| Grouper 3 (QMA0267) | Ib | 261 | | + | + | - | - | - | + | - | + | + | + | + | - | - | - | + | - | - | - | + | - | - |
| Grouper 4 (QMA0280) | Ib | 261 | | + | + | - | - | - | + | - | + | + | + | + | - | - | - | + | + | - | - | + | + | - |
| Grouper 5 (QMA0281) | Ib | 261 | | + | + | - | - | - | + | - | + | + | + | - | - | - | - | - | - | - | - | - | - | - |
| Grouper 6 (QMA0368) | Ib | 261§ | | + | + | - | - | - | + | - | + | + | + | - | - | - | - | - | - | - | - | - | - | - |
| Grouper 7 (QMA0369) | Ib | 261 | | + | + | - | - | - | + | - | + | + | + | - | - | - | - | - | - | - | - | - | - | - |
| Javelin grunter (QMA0268) | Ib | 261 | | + | + | - | - | - | + | - | + | + | + | - | - | - | - | - | - | - | - | - | - | - |
| Javelin grunter (QMA0287) | Ib | 261 | | + | + | - | - | - | + | - | + | + | + | - | - | - | - | - | - | - | - | - | - | - |
| Catfish (QMA0271) | Ib | 261 | | + | + | - | - | - | + | - | + | + | + | + | - | - | - | - | - | - | - | - | - | - |
| Catfish (QMA0290) | Ib | 261 | | + | + | - | - | - | + | - | + | + | + | + | - | - | - | - | - | - | - | - | - | - |
| Mullet (QMA0274) | Ib | 261 | | + | + | - | - | - | + | - | + | + | + | + | - | - | - | - | - | - | - | - | - | - |
| E.S ray 1 (QMA0275) | Ib | 261 | | + | + | - | - | - | + | - | + | + | + | - | - | - | - | - | - | - | - | - | - | - |
| MW ray 2 (QMA0276) | Ib | 261 | | + | + | - | - | - | + | - | + | + | + | - | - | - | - | - | - | - | - | - | - | - |
| Estuary ray 1 (QMA0277) | Ib | 261 | | + | + | - | - | - | + | - | + | + | + | - | - | - | - | - | - | - | - | - | - | - |
| Esturay ray 5 (QMA0320) | Ib | 261 | | + | + | - | - | - | + | - | + | + | + | - | - | - | - | - | - | - | - | - | - | - |
| **Crocodile** | | | | | | | | | | | | | | | | | | | | | | | | |
| Crocodile (QMA0336) | Ia | 23 | 23 | + | + | - | - | - | + | - | + | + | + | + | - | - | - | - | + | - | - | + | - | + |
| **Human** | | | | | | | | | | | | | | | | | | | | | | | | |
| COH1/REF (QMA0370) | III | 17 | 17 | + | + | - | - | - | + | - | + | + | + | + | - | - | - | - | + | - | - | + | - | + |
| Human foot (QMA0355) | Ia | 23 | 23 | + | + | - | - | - | + | - | + | + | + | + | - | - | - | - | + | - | - | + | - | + |
| Female genital (QMA0357) | Ia | 23 | 23 | + | + | - | - | - | + | - | + | + | + | + | - | - | - | - | + | - | - | + | - | + |
| **Bovine** | | | | | | | | | | | | | | | | | | | | | | | | |
| Cow (QMA0306) | III | 67 | 67 | + | + | - | - | - | + | - | + | + | + | + | - | - | - | + | + | - | - | + | - | + |
| **Dog** | | | | | | | | | | | | | | | | | | | | | | | | |
| Dog (QMA0300) | V | 1 | 1 | + | + | - | - | - | + | - | + | + | + | + | - | - | - | - | + | - | - | + | - | + |
| **Cat** | | | | | | | | | | | | | | | | | | | | | | | | |
| Cat (QMA0303) | V | 1 | 1 | + | + | - | - | - | + | - | + | + | + | + | - | - | - | - | + | - | - | + | - | + |

* Hydrolysis (enzymatic tests)
† Fermentation

***Notes: ST-261 first reported in the reference strain: ATCC51487 (KUW) isolated from a Nile Tilapia (Evans et al. 2008). § SLV: single locus variant (1 allele is either novel or in a first time seen combination; ES: Eastern Shovelnose ray; MW: Mangrove Whiptail ray.***

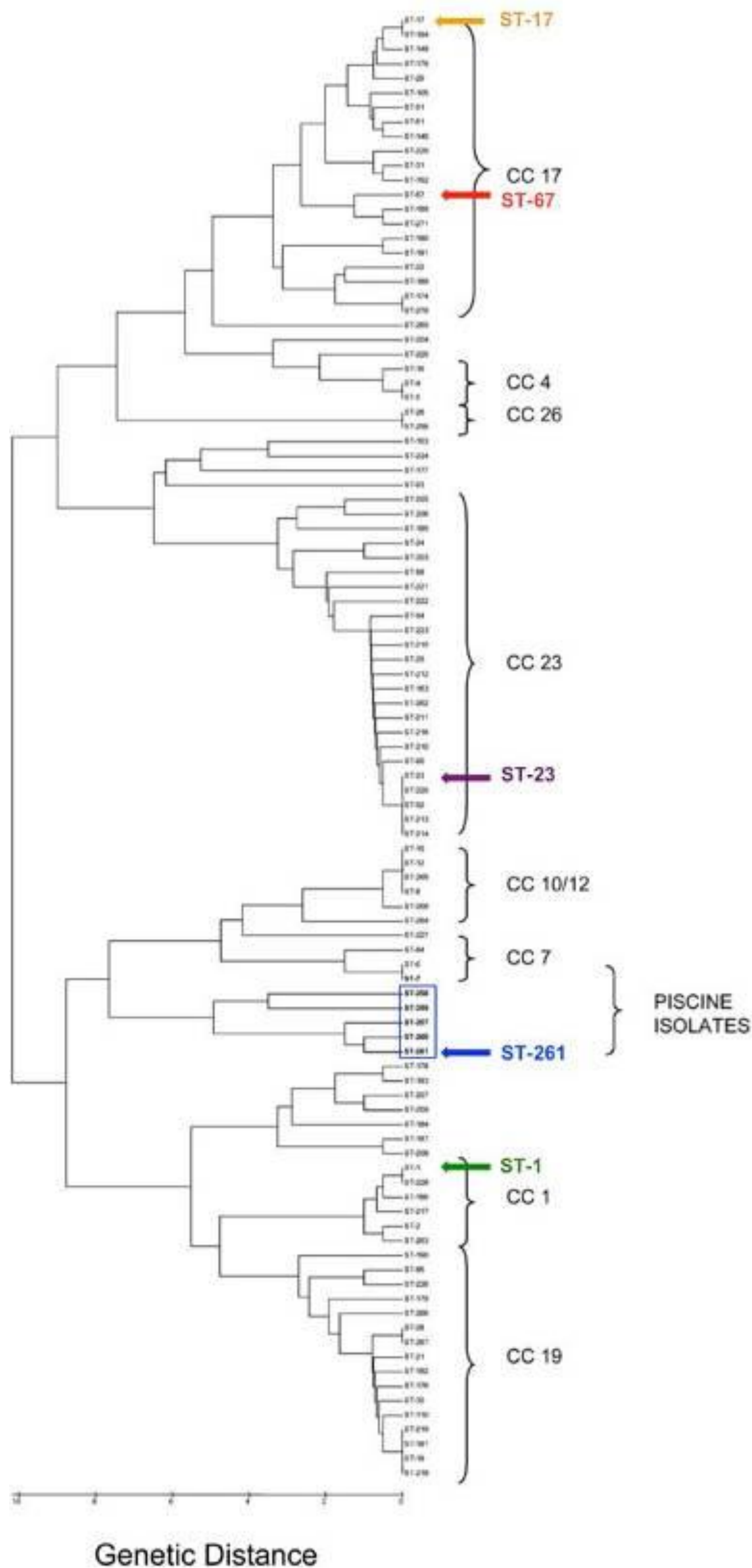


**Figure 6.** Dendrogram illustrating the phylogenetic relationship of the piscine isolates to human and bovines isolates. Clonal complexes (CC) are indicated, as are the STs of the piscine isolates. Note: figure modified from (Evans *et al*. 2008).

Contrary to the piscine isolates, the crocodile strain, QMA0336 (336), isolated on a farm in the Northern Territory (Darwin) from a case of necrotizing fasciitis in captive juvenile *Crocodylus porosus* (Bishop *et al*. 2007) is unrelated the ST 261 group. It was identified as ST-23, serotype Ia, as were the two human isolates included in our study, collected from two cases of infection in Townsville, QMA0355 (355) and QMA0357 (357). ST-23 is a major successful clone identified worldwide and known to have a very wide host range (Brochet *et al*. 2006). It has also been previously reported in juvenile crocodiles and involved the same pathology, but establishing an unambiguous human origin remains to be confirmed (Bishop *et al*. 2007).

COH1 (QMA0370), a clinical isolate from a case of fatal infant septicaemia belongs to the hypervirulent ST-17 lineage (Tettelin *et al*. 2005). COH1 is closely related to the cow isolate (QMA0306, ST-67), as both share four alleles in their allelic profile (Table 21). Both COH1 and the cow isolate cluster in the same clonal complex 17 (Figure 6). Both dog (QMA0300) and cat (QMA0303) belong to the same ST-1, sharing two alleles (*pheS* and *sdhA*) with ST-17 (COH1) and ST-67 (QMA0306) suggesting a possible relationship with human and cow isolates.

**Table 21.** Corresponding allelic profile for each of the sequence type (ST)

| **Serotype (n)*** | **ST** | **Allelic profile** | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | ***adhp*** | ***pheS*** | ***atr*** | ***glnA*** | ***sdhA*** | ***glck*** | ***tkt*** |
| **Ib (16)** | **261** | **54** | **17** | **31** | **4** | **26** | **25** | **19** |
| **V (1)** | **67** | **13** | **1** | **1** | **13** | **1** | **1** | **5** |
| **Ia (3)** | **23** | **5** | **4** | **6** | **3** | **2** | **1** | **3** |
| **V (1)** | **17** | **2** | **1** | **1** | **2** | **1** | **1** | **1** |
| **III (2)** | **1** | **1** | **1** | **2** | **1** | **1** | **2** | **2** |

Single locus variant (SLV) is when one allele is either novel or it is the first occurrence of a particular allele combination. Only one SLV was found amongst the Australian fish isolates, in the *glnA* locus sequence of QMA0368 isolated from grouper 6 (*E. lanceolatus*). In order to submit the novel allelic combination of QMA0368 to the MSLT database, the variant was confirmed by Sanger sequencing Both read, gln368_1 (PCR1) and gln368_2 (PCR2) were identical for our region of interest, thus confirming that the polymorphism detected in *glnA* for QMA0368 is correct. This novel allele is closer to glnA_4 (99.8% identity), which corresponds to the *glnA* allele number found in ST-261.

***Capsular Polysaccharide Sequence analysis of various GBS isolates***

Whilst the MLST at the global level tells us that all piscine isolates belong to the same sequence type (ST 261), it does not give us much information on the genomic variability amongst these isolates at the local scale (Australia). Thus, the plasticity of the variable regions within the capsular polysaccharide operon was analysed. Comparative sequence analysis of their *cps* locus revealed that their serotype was also consistent, as all have been

identified as serotype Ib (Table 20, 22). Differences in size of all the *cps* full sequences are summarized in Table 22 and Figure 7.

**Table 22.** Capsular polysaccharide sequence (cps) size of each isolate

| Isolate<br>Ref. | Host<br>Common Name | *cps* full sequence size (bp) | ST | Serotype |
|---|---|---|---|---|
| QMA0284 | Grouper 1 | 15616 | 261 | Ib |
| QMA0285 | Grouper 2 | 15616 | 261 | Ib |
| QMA0267 | Grouper 3 | 15616 | 261 | Ib |
| QMA0280 | Grouper 4 | 15616 | 261 | Ib |
| QMA0369 | Grouper 7 | 15616 | 261 | Ib |
| QMA0268 | Javelin grunter | 15616 | 261 | Ib |
| QMA0287 | Javelin grunter | 15616 | 261 | Ib |
| QMA0271 | Catfish | 15616 | 261 | Ib |
| QMA0290 | Catfish | 15616 | 261 | Ib |
| QMA0274 | Mullet | 15620 | 261 | Ib |
| QMA0275 | E.S ray | 15616 | 261 | Ib |
| QMA0276 | M.W ray | 15616 | 261 | Ib |
| QMA0277 | Estuary ray 1 | 15616 | 261 | Ib |
| QMA0320 | Estuary ray 5 | 15616 | 261 | Ib |
| QMA0281 | Grouper 5 | 13070 | 261 | Ib |
| QMA0336 | Crocodile | 15722 | 23 | Ia |
| QMA0355 | Human foot | 15722 | 23 | Ia |
| QMA0357 | female genital | 15722 | 23 | Ia |
| QMA0370 | COH1 | 15602 | 17 | III |
| QMA0306 | Cow | 15487 | 67 | III |
| QMA0300 | Dog | 17157 | 1 | V |
| QMA0303 | Cat | 17157 | 1 | V |

There was a high level of conservation amongst the different isolates, with the regions *cpsA* to *cpsD*, *cpsF-cpsG*, *cpsL*, and *neuB* to *neuA* conserved amongst all isolates (Figure 7). The *cps* locus organisation is similar in all piscine isolates with the exception of QMA0281 (grouper 5), which is missing a region of approximately 2,550bp encompassing part of *cpsB, cpsC, cpsD* and part of *cpsE*. The isolates QMA0274 (mullet) and QMA0368 (grouper 6) also harbour minor differences in *cpsH* and *cpsE* and *H* respectively. The crocodile isolate is the only "marine" (non-terrestrial) related isolate to carry a different serotype (Ia). Interestingly, the *cpsJK* region of the QMA0336 (crocodile) isolate (serotype Ia) is the only significant variable region differing in the *cps* locus of the piscine isolates (serotype Ib), which confirms the close relationship of serotype Ia and Ib, as previously established in the literature.

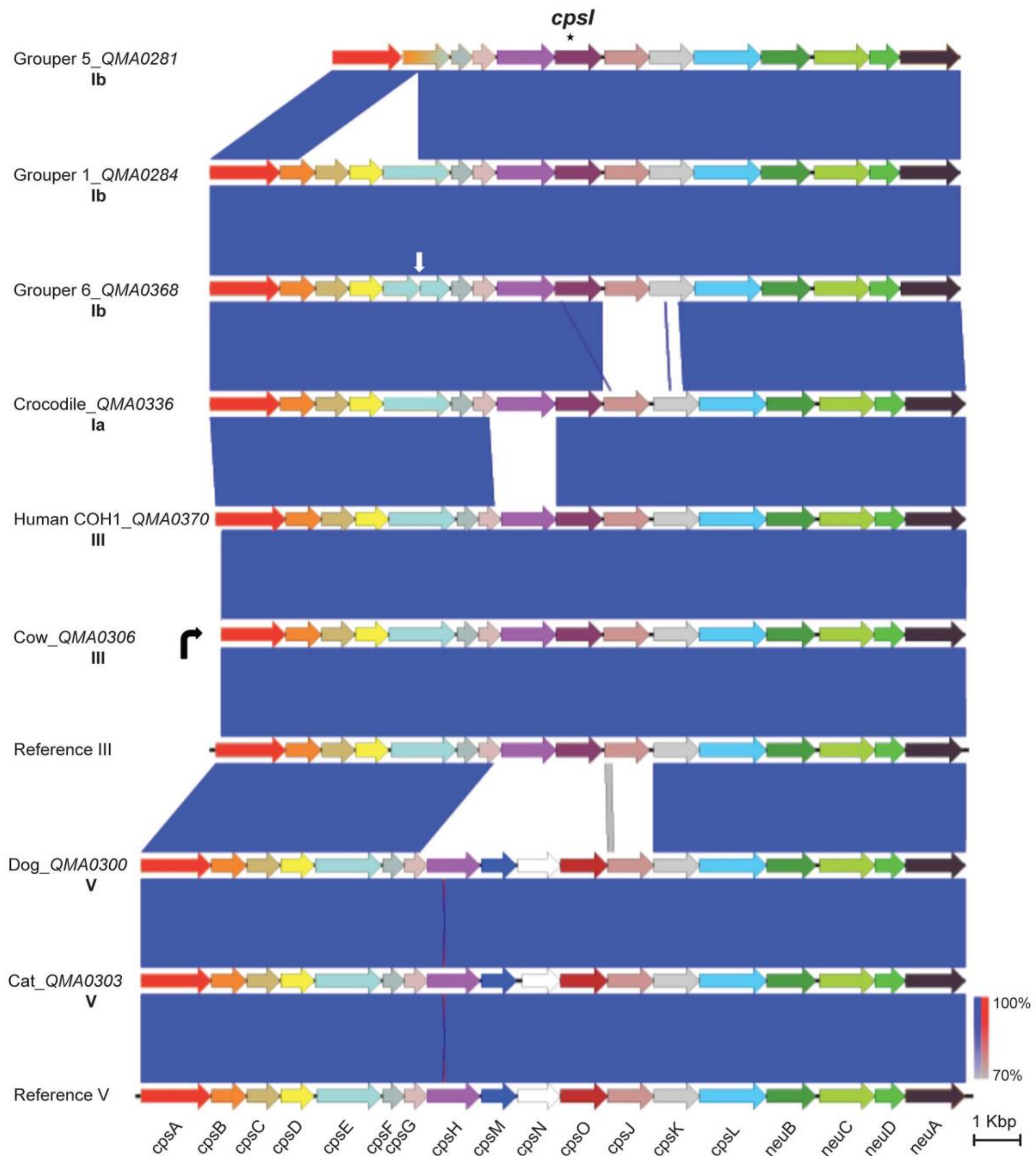


**Figure 7.** Comparison of the genetic organisation of the different polysaccharide synthesis operons identified among the *S. agalactiae* isolates studied. Each sequence is compared to the sequence located below. The nucleotide sequence identity between regions is indicated by a spectrum of blue/red to gray colours depicting a range of 100-70% identity. Each gene of the polysaccharide synthesis operon is shown in a different colour. Of note, a region encompassing part of *cpsB*, *cpsC*, *cpsD* and part of *cpsE* is missing in grouper 5 (QMA0281), resulting in a chimeric fusion gene (colour gradient). White arrows indicate site of insertion (frame shifts) and black arrow the missing starting region of *cpsA* (306). *CpsM, N* and *O* are only present in serotype V (300 & 303). *CpsI* (not shown on the legend) is located just after *cpsH* for all the other isolates (see black star). Figure produced with EasyFig (Sullivan *et al*. 2011).

It is likely that QMA0336 is closely related to the human strains included in this study (QMA0355 and QMA0357), as they fall into ST-23 and serotype Ia, which could be the

result of a recent transmission from human to crocodile. However, these hypotheses remain to be confirmed by whole genome comparative analysis.

Serotype III QMA0306 (cow), serotypes V QMA0300 (dog) and QMA0303 (cat), are nearly identical from a structural point of view to the previously characterized Serotypes III (Glaser *et al*. 2002) and V (Tettelin *et al*. 2002). The major differences between these two serotypes are different alleles for the *cpsH* and *cpsJ* genes, and the replacement of *cpsI* by *cpsMNO* in the serotype V. Overall, all isolates belonging to the same sequence type (ST) had the same serotype, although different serotypes can occur within the same ST (Evans *et al*. 2008).

***Analysis of cps sequence polymorphisms amongst the piscine isolates***

The structural analysis of the *cps* locus of QMA0281 (grouper 5) revealed a significant deletion of approximately 2,550bp of its capsular operon in comparison to the other piscine isolates. We ruled out a possible misassembly of this region by confirming that no sequencing reads were obtained for this region. When looking at the mapping visualisation of the NEM316 capsular region used as a reference template, and mapped reads for QMA0281 (grouper 5) and QMA0280 (grouper 4), where the region is intact (Figure 8), reads are clearly absent from QMA0281, which confirmed a true deletion. Besides this deletion, the rest of the cps sequence was identical when compared to the representative sequence QMA0284, and likely represents a single mutation event.

**Figure 8.** Read mapping visualisation of the NEM316 capsular region for QMA0281 and QMA0280. Reads for QMA0281 are shown in red and reads for QMA0280, in green. There are no reads mapped onto this region for QMA0281 (see in between blue brackets). Of note, pairs of reads are also linking both edges of the missing region indicating this is a true deletion.

Sequence polymorphism analysis of the capsular polysacharride synthesis sequences of all piscine isolates using QMA0284 (grouper 1) as a reference revealed some single nucleotide polymorphisms (SNPs) and insertions (Table 23) which enabled clustering of the isolates. *Cps* loci of isolates from groupers 1 through 4 (QMA0284, 285, 267, 280), grouper 7

(QMA0369), the two javelin grunter (QMA0268, 287) and the two catfish (QMA0271, 290) were identical and formed one cluster. Two SNPs occured in the isolates collected from rays: QMA0275, Eastern shovelnose ray (*Aptychotrema rostrata);* QMA0276, mangrove whiptail ray (*Himantura granulate*); QMA0277 and QMA0320, both from the Estuary rays 1 & 5 (*Dasyatis fluviorum*). These two SNPs are non-synonymous and result in an amino acid change, with an isoleucine (484) being replaced by a valine in *cpsC,* and with a threonine (484) being replaced by a proline in *cpsE*. These reults place the ray isolates in a single cluster, distinct from the grouper isolates. The strains QMA0274 (mullet) and QMA0368 (grouper 6) harbour some small insertions in their *cps* locus. One insertion occurs at position 4,531bp of QMA0368, with two extra thymines, causing a frameshift in the *cpsE* gene. Another insertion occurs at position 6,531bp with an adenine and a thymine inserted and causing a frameshift in the *cpsH* gene. Interestingly, in QMA0274, the same position in the *cpsH* gene presents a similar insertion, with the nucleotides ATAT being inserted instead of AT. Of note, it will be necessary to confirm by PCR both insertions occurring in the *cpsH* gene as they occur in a TA multiple repeat region. This will require designing specific primers for these regions.

**Table 23.** Sequence polymorphisms analysis of all consensus fish *cps* sequences (except QMA0281) compared with Grouper 1 (QMA0284) used as reference.

| | Coverage / Position | QMA0284 Grouper 1 | QMA0267 Grouper 3 | QMA0268 Javelin Grunter | QMA0271 Catfish | QMA0274 Mullet | QMA0275 ES Ray | QMA0276 MW Ray | QMA0277 Estuary Ray 1 | QMA0280 Grouper 4 | QMA0285 Grouper 2 | QMA0287 Javelin Grunter | QMA0290 Catfish | QMA0320 Estuary Ray 5 | QMA0368 Grouper 6 | QMA0369 Grouper 7 | Translation | *cps* Locus |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Reference | Consensus: *Complete* | *Complete* | *Complete* | *Complete* | *Complete* | *Complete* | *Complete* | *Complete* | *Complete* | *Complete* | *Complete* | *Complete* | *Complete* | *Complete* | | |
| *SNP* | 2.681 | A | | | | | G | G | G | | | | | G | | | Iso$_{ATC}$ -> Val$_{GTC}$ | *cpsC* |
| *SNP* | 3.917 | A | | | | | C | C | C | | | | | C | | | Thr$_{ACT}$ -> Pro$_{CCT}$ | *cpsE* |
| *Insertion* | 4.351 | : | | | | | | | | | | | | | T T | | [stop AA258] (norm. 462 AA) | *cpsE* |
| *Insertion* | 6.531 | : | | | | ATAT | | | | | | | | | | | [stop AA187] (norm. 403 AA) | *cpsH* $_{(1)}$ |
| *Insertion* | 6.531 | : | | | | | | | | | | | | | A T | | [stop AA194] (norm. 403 AA) | *cpsH* $_{(1)}$ |

Abbreviations: SNP: single nucleotide polymorphism; Iso: isoleucine; Val: valine; Thr: threonine; Pro: proline; AA: amino acid; *cpsC*: putative capsular polysaccharide chain length regulator; *cpsE*: glucose-1-phosphate transferase (load sugar); *cpsH*: putative polysaccharide repeating unit polymerase.

In terms of the evolution, these differences could suggest an ongoing evolving process, potentially related to host adaptation or pathogenesis. It is intriguing that the strains QMA0274 and QMA0368, isolated from 2 different species, at 2 different locations and time points, respectively (mullet from Cairns in 2009) and (grouper 6 from Topsy creek, Gulf of Carpentaria in 2010) share a common defective mutation at the same position in *cpsH*. If these isolates originated from a common ancestor as it appears from our preliminary analysis,

it would be interesting to determine whether they are undergoing positive selection as a result of specific selective pressures, driving them to diverge from the rest of the isolates. It may also be that there has been translocation of the infection from Cairns to Carpentaria, possibly through wild fish movement, or through transport of fish as bait.

***Discussion and Conclusions***

Multilocus sequence typing (MLST) revealed that all of the piscine GBS isolates from Australia fell into the ST-261 group. This is a one of a number of related strain types that are commonly found in fish (Evans *et al*. 2002, 2008, 2009). Amongst the fish sequence types, ST-261 has only been identified previously in tilapia, and is identical to the type strain that was recovered from Nile tilapia (*Oreochromis niloticus*) in Israel and subsequently translocated to the US and Brazil. This is intriguing, since of all of the fish sequence types occurring globally, only ST-261 is present in Australia to date, a strain associated with tilapia. There are two commonly known and well-established species of tilapia in Northern Queensland; the Mozambique mouth-brooder (*Oreochromis mossambicus*), and the black mangrove cichlid *(Tilapia mariae)*. The convict cichlid (*Cichlasoma nigrofasciatum*), pearl cichlid and jewel cichlid are also found in small isolated pockets. These five introduced cichlid fish species are all declared and noxious exotic pest fishes in Queensland, and represent five of over 100 cichlid fish species that have been imported into Australia for the aquarium trade and have subsequently been released into the wild and have established breeding populations.

The five above-mentioned tilapia species have been introduced into North Queensland on several occasions since the 1970's. Populations of all five types and reported hybrid species, exist in the Cairns, Atherton Tablelands, and Townsville regions and further south, where several species and hybrid species are also present in many Northern rivers (Mather & Arthington, 1991). Whilst it cannot be proven definitively, it is possible, that this strain type (ST-261) may have been introduced, into Australia, along with the various tilapia hybrid species, or other cichlids, and has since established amongst the Australian aquatic animal life in the region. This requires further research and investigation.

One new MLST allele was discovered in a grouper isolate. As the genes used for MLST occur in the core genome and are under positive selective pressure, they evolve very slowly. At this time we are unsure of mutation rates in GBS, but a single allele change amongst the ST-261 type suggests a relatively recent introduction, as there has been insufficient time for major allelic variation.

The preliminary whole genome sequence comparison and *cps* sequence typing corroborate the MLST. Moreover, it is evident that the fish isolates have not occurred as a recent transfer from either human or terrestrial animal sources, since the genomes are smaller in the marine isolates, and serotypes are different. This is reassuring from a public safety perspective as these sequence types have not been found causing disease in humans or terrestrial animals. The substantial gene loss amongst the marine isolates may include loss of genes essential for colonization of mammals, although this will require complete annotation and further analysis of the genomes.

The *cps* genotyping provided some further resolution of possible strain movement and evolution within the aquatic animals. Identical point mutations have been found in all of the isolates from stingrays suggesting passage amongst these animals. Moreover, four of the grouper, the javelin grunters and the catfish shared identical *cps* genotype isolates. Bearing in

mind the plasticity of the *cps* operon in *Streptococcus* sp., this strongly implicates a shared source of infection amongst these fish.

**Conclusion**

The complete genomes of 23 GBS isolates were sequenced and a preliminary assembly completed. Final assembly and full annotation is ongoing and will be completed in the future, as it is beyond the scope of this research project. From the assembled genomes, the strains were typed by MLST and serotyped. All fish isolates from Australia belong to ST-261, a type previously found in Nile Tilapia from Israel (Evans *et al.* 2008). The marine strains were not closely related to terrestrial animal or human isolates (the genomes are 200-300 kb smaller) and have therefore not been recently transferred from terrestrial sources. They are therefore unlikely to pose a food safety issue as these sequence types occur in food fish overseas and have not been associated with human illness. Our data suggest a possible potential of introduction of the strain with tilapia species to North Queensland sometime during the last few decades.

## 6.6 Development of injection and immersion challenge models in Queensland grouper *E. lanceolatus* and pathology analysis

### 6.6.1 Methods

For experiments 1, 2 & 3 in this section, juvenile Qld grouper (*E. lanceolatus*) were air-freighted from the Northern Fisheries Centre (NFC), in Cairns to the University of Queensland in January, 2011. Fish were acclimated for four weeks in three distinct recirculating systems, two quarantine aquaria in an isolated room, and one holding system, all free from disease.

A health test by histopathology examination was done on a subsample of 30 juvenile grouper to assess overall health status, prior to use in experimental trials. Fish were found to be free of disease. A single strain (QMA0285) of *S. agalactiae* was then used in both experiments 2 & 3 (the injection and immersion challenge trials, respectively) in juvenile grouper.

**Experiment 1. Pilot study to determine virulence of *S. agalactiae* in Qld grouper (*E. lanceolatus*)**

A pilot trial was done, to establish virulence of two strains of *S. agalactiae* (Group B *Streptococcus* or GBS), isolated from wild adult Qld grouper in 2010, by injection into healthy juvenile grouper.

A 150 L cylindrical tank was set up in a confined recirculating marine quarantine aquarium in the Ritchie building at UQ. The tank was equipped with an Eheim water pump, 2 electrical air pumps and one heater. Four fish were transferred to the isolated tank when water temperature reached 28°C and salinity 35 ppt.

***Bacterial strains used in study***

Two strains of *S.agalactiae*, previously isolated from dead wild Qld grouper, were used in the pre-injection experiment 1 (Table 24). They were retrieved from stock (held at -80°C) and routinely cultured on sheep blood agar (SBA) (Oxoid) for 48hr at 28°C. From pure culture, a single colony forming unit (cfu) from each strain was inoculated into 5ml Todd Hewitt Broth (THB) and incubated overnight at 28°C with 100 rpm agitation.

**Table 24.** *S. agalactiae* strains used in this study.

| Strain No. | Host | Year | Geographical Isolation | Organ of isolation |
|---|---|---|---|---|
| QMA0280 | Qld Grouper | 2010 | Townsville | Kidney |
| QMA0285 | Qld Grouper | 2010 | Cairns | Head Kidney |

***Genomic DNA extraction from bacterial broth culture***

Aliquots (100µl) from each overnight 5 ml starter culture (QMA0280 & QMA0285) were collected, placed in PCR tubes (200 µl, Scientific Specialities Inc) and centrifuged for 2 min at 12,100 x *g*, supernatant removed and pellets resuspended in 50 µl of UltraPure DNAse, RNAse free water (Gibco). Bacteria were lysed by heating for 15 min at 95°C (Eppendorf) and the solution used as DNA template for PCR analysis.

***Polymerase chain reaction (PCR) for S. agalactiae identity confirmation using AgaF/AdyR***

To confirm the presence of *S. agalactiae*, PCR analysis was conducted on ten-fold serial dilutions ($10^0$ to $10^2$) of DNA template using the AdyF/ AdyR primer set as described above.

*Preparation of bacterial inocula*
The remaining 4.9 ml of each culture described above were pelleted by centrifugation 5 min at (4000 x *g*) and washed twice in PBS. Pellets were resuspended in an appropriate volume of PBS until a final optical density of 1.0 was reached at $A_{600}$ (absorbance at 600 nm wavelength). An OD = 1 for GBS was estimated to be between 1 to 3 x $10^8$ cfu $ml^{-1}$. Once OD=1 was fixed for both strain, they were subjected to an ten-fold dilution in sterile PBS to get to $10^7$ cfu $ml^{-1}$ and 100 µl of that dilution was injected per fish (approximately $10^6$ cfu $fish^{-1}$). A similar approach was used for the immersion challenge, ie. determined concentrated preparations were diluted in a 10 L bucket, to reach the desired final concentration.

*Viable count using tryptic soy agar (TSA) plates*
To confirm the final challenge doses accurately (retrospectively) viable counts were performed on successive serial 10-fold dilutions in sterile PBS of each challenge suspension. 100 µl from each dilution were plated on TSA and incubated for 48 h at 28°C.

**Experiments 2 & 3: Replicated challenge models to determine infectivity by injection and immersion challenge trials**

*Experimental fish and husbandry*
For the purposes of both experimental challenge trials 2 & 3, juvenile Queensland grouper (*E. lanceolatus*), weighing approximately 30 g, were obtained from the Northern Fisheries Centre in Cairns, air freighted to UQ and held in full strength sea water at 35 ppt salinity in 100 L fiber glass aquaria. Aquaria were organized into two banks (replicates 1 and 2), consisting of eight tanks per bank. Both banks (replicates 1 & 2) were each supplied by a separate recirculation system, consisting of 1 x 200 L sump, 50 L bio-filter, and a protein skimmer.

For both experiments 2 and 3, fish were randomly assigned to each tank, such that there were eight individual fish per tank. Remaining fish were maintained in a separate holding system located in a different room, comprising eight tanks (160 L each), 2 x 200 L sumps, 2 x 50 L bio-filters, and a protein skimmer. The temperature of all systems was maintained at 28°C by three heating and cooling systems, with a recirculation rate of approximately 120 L per hour. Fish were fed on a maintenance diet consisting of a commercial 4 mm floating pellet (Ridley Aqua Feed, Narangba, Queensland) once a day, equivalent to 5% body weight. A 15% water exchange was performed each week. Water quality was checked each week for ammonia, nitrite and pH. Fish were allowed to acclimatize for a period of four weeks. Survival analysis was determined for two different exposure routes, intra-peritoneal injection and immersion challenge as follows.

**Experiment 2: Injection challenge trial**
Six treatments ($10^4$, $10^5$, $10^6$, $10^7$, $10^8$, $10^9$ cfu $ml^{-1}$) were prepared by pathogen dose titration, using serial ten fold dilutions of a strain of *S. agalactiae* isolated from wild Queensland grouper (QMA0285). Eight fish were allocated per tank, per treatment (in duplicate), using replicates 1 & 2 of the marine quarantine system. After a period of acclimation, fish were anaesthetised with AQUI-S in accordance with the manufacturer's directions for marine finfish (20 mg $L^{-1}$). The two replicate cohorts of eight fish per treatment were challenged by intra-peritoneal injection (ip) with 100 µl of the standardised ten-fold dilution of *S. agalactiae*, giving a final inoculum per fish ranging from $10^3$ to $10^8$ cfu $fish^{-1}$. Eight fish made up the negative control group. These fish were injected with 100 µl of sterile PBS solution, and were allocated to a separate tank, located in a separate recirculation system.

**Experiment 3: Immersion challenge trial**
In the immersion challenge trial, replicates 1 & 2 of the marine quarantine system at UQ were again used for this experiment. Each replicate system, had three treatment groups, consisting of immersion of fish in seawater inoculated with low, medium and high doses of *S. agalactiae*: $10^4$, $10^5$, and $10^6$ cfu $ml^{-1}$ respectively. Each treatment group consisted of 8 fish, allocated to a single tank, within a flow-through system (replicates 1 & 2). Two separate sentinel control groups of fish (unchallenged) were maintained, in two separate tanks, (one tank within each separate replicate system 1 & 2), to test whether flow-through of water could transmit the disease.

Only one positive control group was used, because virulence by injection of GBS had already been demonstrated in juvenile Queensland grouper (*Experiment 1: Pilot study to determine virulence of S.agalactiae in Qld grouper* and *Experiment 2 Injection challenge trial*). The positive control group consisted of eight fish in an allocated tank within the flow-through system of replicate 1. These fish were injected intra-peritoneally (at $10^6$ cfu $fish^{-1}$) using a sub-sample of the same challenge inoculum used for the immersion trial, as a positive control to test if the bacteria could be shed into the water.

A group of eight fish were immersed in seawater under the same experimental conditions and placed in the holding system as a negative (handling) control.

Briefly, the immersion challenge was performed as follows: 2 ml of an overnight THB-l culture was inoculated in 200 ml fresh medium (0.1%) and incubated at $28^0$C for 24 h with shaking at 100 rpm. Cohorts of eight grouper per treatment were maintained outside of their original tank in aerated 10 L bucket and exposed separately for 60 min to one of three serial 10-fold dilutions of GBS strain QMA0285 ranging from $10^4$ to $10^6$ cfu $ml^{-1}$. All buckets were covered with a lid to prevent aerosol dispersion of the challenge *S. agalactiae* inoculum. After 1 h, fish were returned to their respective tanks and water from immersion buckets was disinfected (8 ml of sodium hypochlorite 12.5% per 100 L of contaminated water) before being drained. The two sentinel groups were unchallenged and left in their respective tanks (replicates 1 & 2). The eight positive control fishes (replicate 1) were injected as described above.

Mortalities were recorded every 4 h until four consecutive days passed without further mortality. All remaining fish were euthanased on day fifteen, by immersing fish in AQUI-S at a dose rate of 40 mg $L^{-1}$ at the end of the experimental challenge trial.

**Bacteriological analysis and PCR detection of QMA0285 from bacterial cultures**
From pure culture on SBA, QMA0285 showed typical phenotype of GBS. Several cfu from each organ of all sampled fish were collected using a 1µl sterile loop and resuspended in PCR tubes (200 µl, SSI) containing 50 µl of ultra pure DNAse, RNAse free water (Gibco). DNA template was prepared and subject to PCR analysis as previously described. If contamination occurred, only GBS-like bacteria were sampled for PCR. When no growth occurred, regions of the SBA plate were still swabbed for PCR.

For experiments 2 & 3, (for both replicates 1 & 2), two fish from each treatment group (including moribund, and survivor fish) were sampled for bacteriology at UQ. Samples were collected from internal organs (brain, kidney, liver and spleen), dissected out using aseptic techniques, streaked onto SBA, and incubated at 28°C for up to three days. Bacterial isolates

were confirmed as *S. agalactiae* by PCR analysis done on bacterial cultures. At TAAHL, PCR was done on whole organs, for five to seven fish for each treatment group. PCR was done on individual, whole organs including the brain, spleen and kidney, according to methodology outlined previously.

**Histology & histopathology analysis**
All euthanased or dead fish, from each tank, from each treatment group, and each replicate, from both experiments 2 & 3, were sampled for histology. Organs sampled included the gills, eye, brain, heart, head kidney, caudal kidney, spleen, liver, muscle, skin, stomach, intestine, pyloric caecae and swim bladder. Organs were fixed in 4% paraformaldehyde (PFA) for 48 h, tissues rinsed to remove excess PFA and transferred to 70% alcohol. All samples were sent to TAAHL for histological processing, staining and sectioning. Samples were stored at 4 °C until being processed for histology. Sections (5 µm) were cut and stained with haematoxylin and eosin. Special stains including Gram Glynn, Brown & Brenn's Gram stain, and Ziehl-Neelson and were prepared according to Bancroft and Stevens (1992). Each histological tissue section was examined for a minimum of 20 minutes, using a compound Olympus BX51 light microscope. Photomicrographs of pathology lesions were taken using an Olympus UC-30 Cam colour camera using digital AnalySIS software.

A whole fish was considered overall positive, if Gram-Glynn-positive staining cocci-shaped bacteria were detected inside any organ or tissue of a fish, together with pathology indicative of streptococcosis. Gram Glynn stains done on all organs and tissues from each fish, to detect Gram Glynn positive staining cocci, were examined in detail, to contribute to the final diagnosis for each fish.

For the purposes of test comparison (PCR *vs* bacteriology *vs* histology), 3 organs were selected for test comparison, the brain, spleen and kidney. Gram Glynn stains done on these three organs were used for test comparison purposes. These organs were each examined in detail, for each fish, by light microscopy, for a minimum of 20 min per histological slide.

**PCR**
PCR analysis, according to methodology described previously, was done on whole tissues, including the brain, spleen and kidney from each fish, from each treatment group, and from each replicate, from fish from both experiments 2 & 3.

### 6.6.2 Results and Discussion

**Experiment 1: Pre-injection challenge**
Both strains QMA0280 and QMA0285 were confirmed to be *S. agalactiae* by PCR using primer pair AgaF/AdyR (Fig. 9A). QMA0285 was confirmed by PCR before being used in the injection challenge trial (Fig. 9B).

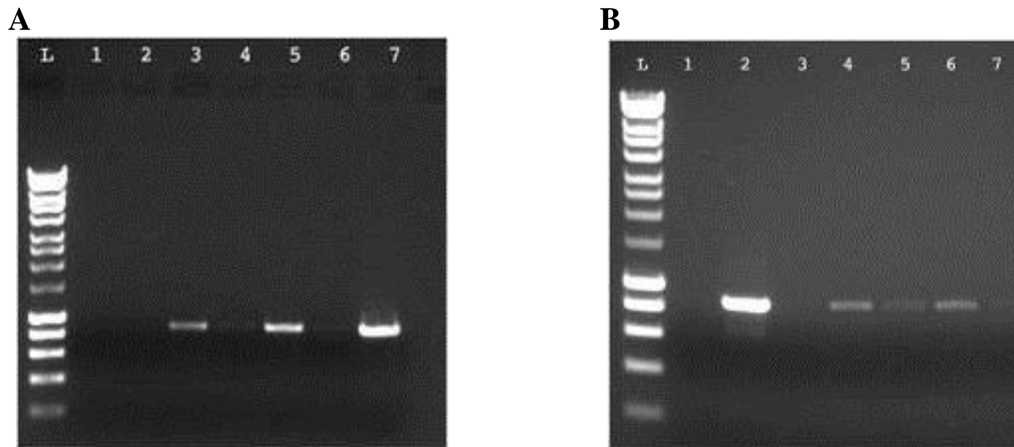


**Figure 9.** *S. agalactiae* identity confirmation by PCR using AgaF/AdyR primer pair. AgaF and AdyR are specific for *S. agalactiae*. For each reaction 3 µL PCR product were mixed with 2µL loading buffer and loaded onto a 1% agarose gel; L: 5 µL markers (HyperLadder, Bioline); **A**. Pre-injection trial. 1: negative control using genomic DNA from *S. iniae*; 2: Template-free control; 3: DNA from strain QMA0280 isolated from Qld grouper (neat); 4: DNA from strain QMA0280 (1/10 dilution); 5: DNA from strain QMA0285 isolated from Qld grouper (neat); 6:DNA from strain QMA0285 (1/10 dilution); 7: positive control using known GBS's DNA from strain QMA0326 (1/100 dilution) isolated from Estuary ray (*Sea World*). **B**. Injection trial. 1: Template-free control; 2: positive control using known GBS's DNA from strain QMA0326 (1/100 dilution) isolated from Estuary ray (*Sea World*); 3: negative control using genomic DNA from *S. iniae* #122 (1/100 dilution)*;* 4: DNA from strain QMA0280 isolated from Qld grouper (neat); 5: DNA from strain QMA0280 (1/100 dilution); 6: DNA from strain QMA0285 isolated from Qld grouper (neat); 7: DNA from strain QMA0285 (1/100 dilution).

To confirm pathogenicity of the strains, two fish were injected with 1.85 x $10^6$ cfu $ml^{-1}$ (strain QMA0285), and two fish with 1.56 x $10^6$ cfu $ml^{-1}$ (strain QMA0280). Mortalities occurred for both bacterial strains used, on day 5 and day 6 post-injection. The first fish died on day 5 (injected with QMA0280) followed by the second fish death, on day 6 (injected with QMA0285). Based on the confirmed virulence of both *S. agalactiae* isolates, it was decided to use strain QMA0285 for subsequent replicated injection and immersion challenge trials.

**Survival curves for the experimental challenge trials 2 & 3**

***Experiment 2: Injection challenge trial***

The results from this study indicated that injection challenge with *S. agalactiae* (strain QMA0285) causes nearly 100 % morbidity and mortality in juvenile Qld grouper within 4 days, at all six doses ranging from $10^3$ to $10^8$ cfu $fish^{-1}$ (Fig.10a). In replicate 1 (Fig.10a), fish began dying after 40 h (1.66 days) post injection (group $10^5$ and $10^6$ cfu $fish^{-1}$); at the other doses, fish died within 78.3 h (3.27 days) except for the higher dose $10^8$ cfu $fish^{-1}$ where fish died after 69.5 h (2.89 days). In replicate 2 (Fig. 10b), the first fish that died was from the highest dose ($10^8$ cfu $fish^{-1}$) at 26 h (1.08 days) post injection, followed by fish from other dose groups. Chronologically, fish from the high dose treatment group all died within 63.5 h (2.64 days), followed by $10^7$ cfu group within 72.5 h (3.02 days), then the group $10^6$, $10^5$ and $10^4$ cfu, which all died after 78.5 h (3.27 days). The last fish to die was from the lowest dose group ($10^3$ cfu $fish^{-1}$), with the last fish dying after 93 h post injection (3.87 days). Only two fish survived (replicate 2, low dose $10^3$ cfu $fish^{-1}$), with only one showing clinical signs of disease.

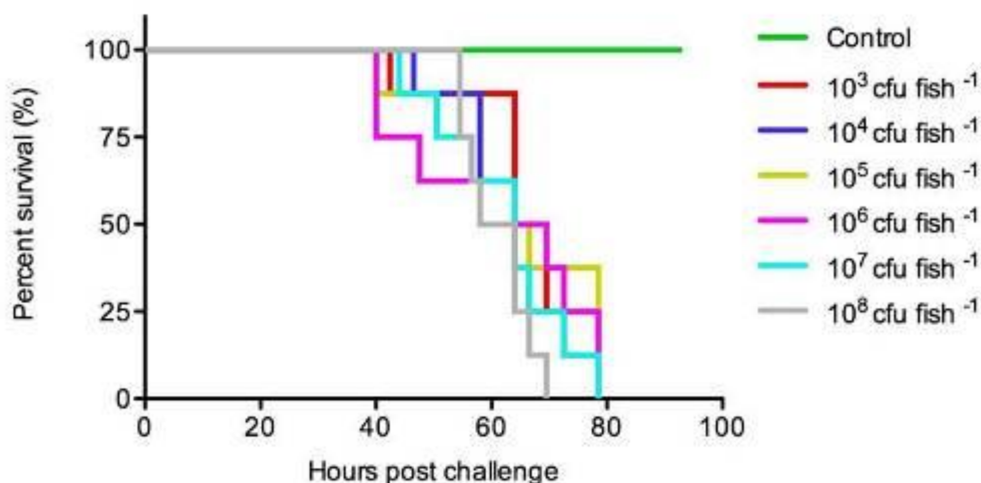


**Figure 10a.** Survival results from the injection challenge trial (replicate 1) using *S. agalactiae* (QMA0285 strain) inoculated into juvenile Queensland grouper *E. lanceolatus,* by intra-peritoneal injection (Kaplan-Meier survival curves). Six doses were used in the injection challenge trial, ranging from $10^3$ to $10^8$ cfu injected per fish. Each cohort of eight fish was injected per treatment. Comparison of survival curves using log-rank (Mantel-Cox) test showed that curves were significantly different and therefore confirmed a dose effect (replicate 1: P=0.0031 and replicate 2: P<0.0001).

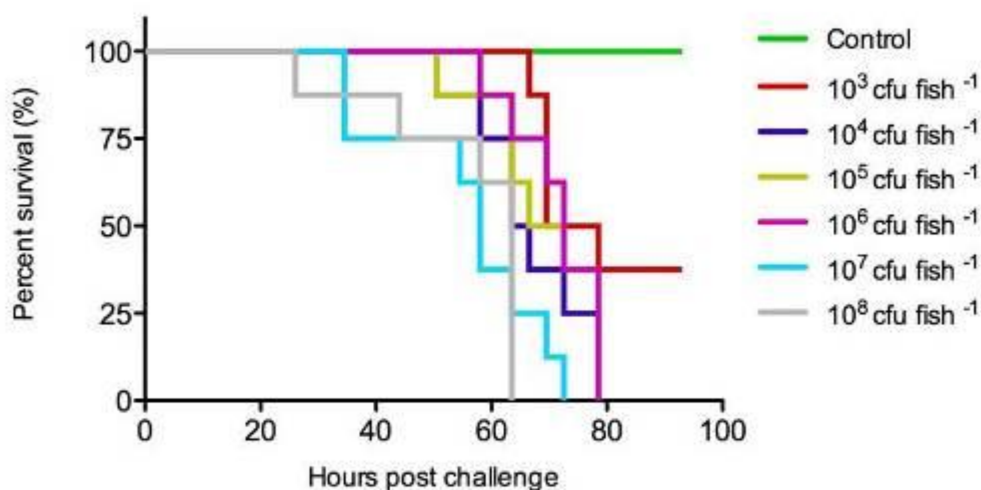


**Figure 10b**. Survival results from the injection challenge trial (replicate 2) using *S. agalactiae* (QMA0285 strain) inoculated into juvenile Queensland grouper *E. lanceolatus,* by intra-peritoneal injection (Kaplan-Meier survival curves). Six doses were used in the challenge trial, ranging from $10^3$ to $10^8$ cfu injected per fish. Each cohort of eight fish was injected per treatment. Comparison of survival curves using log-rank (Mantel-Cox) test showed that curves were significantly different and therefore confirmed a dose effect (replicate 1: $P = 0.0031$ and replicate 2: $P < 0.0001$).

***Experiment 3: Immersion challenge trial***

Results from the immersion challenge trial, showed that in replicate 1 (Fig.11a), there was 100 % survival in the low, medium (not shown on graph) and sentinel groups; 87.5% survival was recorded for the high dose group ($10^6$ cfu ml$^{-1}$) with a single fish dying 169 h post immersion (7 days). A consistent 100 % mortality was recorded in the positive control (injected) group; with the first fish dying 67 h post challenge (2.8 days) and the last one at 92.5 h (3.85 days), thereby confirming the high virulence of this inoculum.

In replicate 2 (Fig. 11b), an 87.5 % survival was monitored in the low dose, with a single fish dying 82 h post challenge (3.42 days); 75 % survival in the medium dose with first fish dying 62 h post immersion (2.6 days) and the second fish at 144 h (6 days); 37.5 % survival in high dose with first fish dying after 73 h (3.04 days) and fifth fish dying last after 165.5 h post immersion (6.89 days). All sentinel fish survived. Immersion challenge lasted for a total of 355 h (14.8 days), with 7.75 consecutive days without mortality (replicate 1) and 7.89 days (replicate 2).

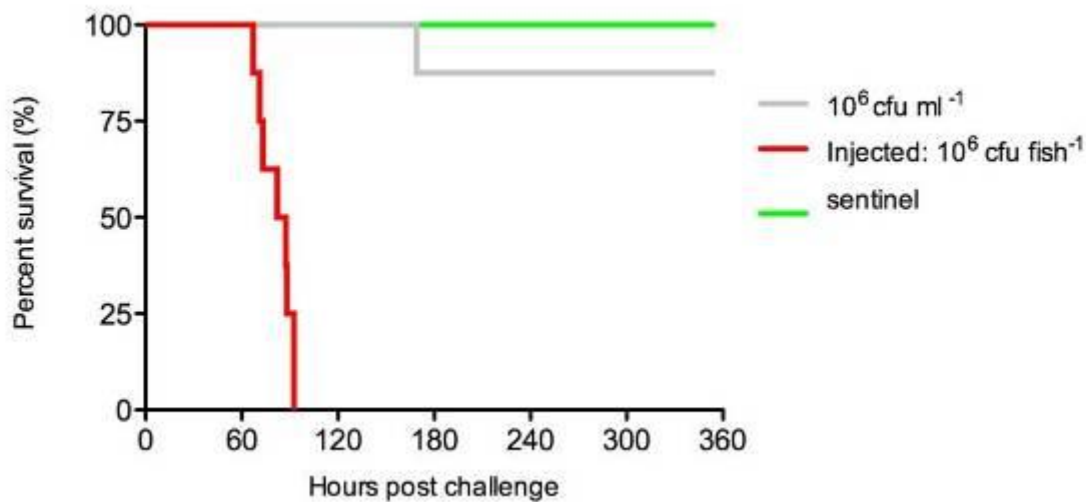


**Figure 11a.** Survival results from the immersion challenge trial (replicate 1), using *S. agalactiae* (QMA0285 strain) for juvenile Queensland grouper *E. lanceolatus* (Kaplan-Meier survival curves). Three doses ranging from $10^4$ to $10^6$ cfu ml$^{-1}$; sentinel fish were unchallenged; eight fish immersed per dose. Comparison of survival curves using log-rank (Mantel-Cox) test showed that curves were significantly different for system 1: $P < 0.0001$ but not significantly different for system 2: $P = 0.2129$.

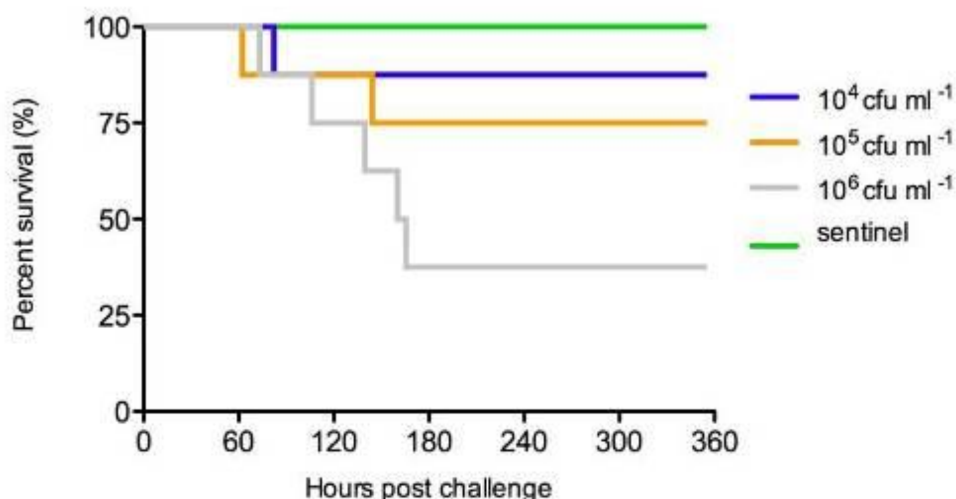


**Figure 11b.** Survival results from the immersion challenge trial (replicate 2), using *S. agalactiae* (QMA0285 strain) for juvenile Queensland grouper *E. lanceolatus* (Kaplan-Meier survival curves). Three doses were used in the challenge trial, ranging from $10^4$ to $10^6$ cfu ml$^{-1}$; sentinel fish were unchallenged; a cohort of eight fish, were immersed per dose. Comparison of survival curves using log-rank (Mantel-Cox) test showed that curves were significantly different for system 1: $P < 0.0001$ but not significantly different for system 2: $P = 0.2129$.

### *S. agalactiae* detection by bacterial culture (with PCR confirmation) at UQ

***Experiment 2: Injection challenge trial***

In the injection challenge trial, strain QMA0285 was successfully recovered on SBA from all organs (except six), for replicate 1, and from all organs except 4 (replicate 2) sampled for bacteriology, for all six treatment groups ranging from $10^3$ to $10^8$ cfu. Results are shown for two fish tested per treatment group (Appendices 3 & 4). PCR, done on bacteria isolates from internal organs, confirmed *S. agalactiae* identity in nearly all cases, except for six organs (replicate 1) and four organs (replicate 2). In terms of specificity, all bacterial isolates were positive using the *S. agalactiae* specific primer pairs AgaF/AdyR. 1 in 100 dilution was necessary for some samples, to react positively (as excess DNA inhibited PCR reaction). The two surviving fish (fish no. 7 & 8), from replicate 2 (injected with the lowest inoculum, $10^3$ cfu fish$^{-1}$), tested negative by bacteriology, but were positive by PCR done on whole tissues and by histopathology analysis (Appendices 3 & 4).

***Experiment 3: Immersion challenge trial***

Similarly for the immersion challenge trial, bacteriology was done on two fish per treatment group, from internal organs. Strain QMA0285 was successfully recovered on SBA for some fish, and PCR (done on bacterial isolates), confirmed *S.agalactiae* identity (Appendices 5 & 6). *S. agalactiae* was successfully isolated from two of the positive control fish (Appendices 5 & 6). Similarly, *S. agalactiae* was successfully isolated from two fish each, from both the high dose and medium dose treatment groups, and confirmed as *S. agalactiae* by PCR (for replicate 2 only) (Appendices 5 & 6). In contrast, no bacteria were isolated from any of the three treatment groups from replicate 1. All negative control treatment groups were negative by bacteriology.

### Bacteriology by Gram stain

It was possible to visualise gram-positive cocci from impression smears made from internal organs (eg. brain, kidney, liver or spleen) from juvenile Queensland grouper suffering from streptococcosis. They were stained with a Gram stain kit and examined by light microscopy.

### Gross external clinical signs of Streptococcosis in juvenile grouper *E. lanceolatus*

***Experiment 2: Injection challenge trial***

In the injection challenge trial, juvenile grouper infected with *S. agalactiae* displayed clinical signs of streptococcosis that varied between fish, both within a treatment group, and between different treatment groups. Fish became anorexic, (depending on the treatment group), but this generally occurred at high doses, and within 24 h post infection. Fish were also lethargic, disorientated and showed erratic swimming, indicating central nervous system involvement. Clinical signs included unilateral or bilateral exophthalmia, opacification of the eye, intraocular haemorrhage, pale gills with petechiae, ascites, dermatitis with hyperaemia and oedema and anal congestion. Clinical signs were typically observed in nearly all treatment groups of the injection challenge trial, with marked clinical signs and altered behaviour seen in fish from the highest dose treatment groups ($10^7$ & $10^8$ cfu/fish).

**Figure 12.** External clinical signs of streptococcosis in juvenile Queensland grouper. **A**. right lateral view of a normal eye (control fish); **B**. right lateral view of an eye with severe exophthalmia; **C**. eye with intra-ocular haemorrhage; **D**. bilateral exophthalmia; **E**. eye opacity; **F**. skin ulceration and dermatitis of the lower jaw caused by *S. agalactiae*; **G**. intra-peritoneal injection point with ulceration, hyperaemia and congested anus; **H**. skin ulceration and haemorrhage at the base of the pectoral fin; **I**. skin lesion with ulcerative dermatitis, dermal oedema, and haemorrhage on the flank (immersion challenge, high dose); **J**. dermal oedema on the flank near the caudal fin; **K**. pale gills with petechial hemorrhages; **L**. normal gills from a control fish (absence of petechial hemorrhages).

***Experiment 3: Immersion challenge trial***

In the immersion challenge trial, juvenile grouper (depending on the treatment group) developed either unilateral or bilateral exophthalmos (Fig.12B & 12D) compared to control fish (Fig.12A). Some fish showed opacification of the eye (Fig.12E). The eyes of fish also displayed intraocular haemorrhage, visible in fish with severe exophthalmia (Fig.12C). Other organs displaying haemorrhage included the gills and skin. The gills were pale, with multifocal petechial haemorrhages visible along the gill filaments (Fig.12K), compared to normal gills (Fig.12L). The skin showed oedema (Fig.12J), ulceration with hyperaemia, often on the mouth or lower jaw, at the base of the pectoral fin, around the anus, or on the flank at the point of inoculation (Figs. 12F, 12H and 12K). Many challenged fish also showed hyperaemia or congestion around the anus or genital papilla (Fig. 12G).

**Internal signs of streptococcosis in juvenile grouper *E. lanceolatus* (experiments 2 & 3)**

In experiment 2, moribund or dead fish had an empty stomach when dissected, with an enlarged gall bladder (Fig.13C). The stomach and intestine of some fish were filled with a

yellowish fluid, indicating enteritis. Fish displayed signs of septicaemia, including splenomegaly, with haemorrhage of the spleen and kidney (Fig.13D). Ascites was commonly observed in dissected moribund fish (Fig.13A), compared to negative control fish (Fig.13B). Juvenile grouper frequently had a protruding, hyperaemic anus, often accompanying the ascites.

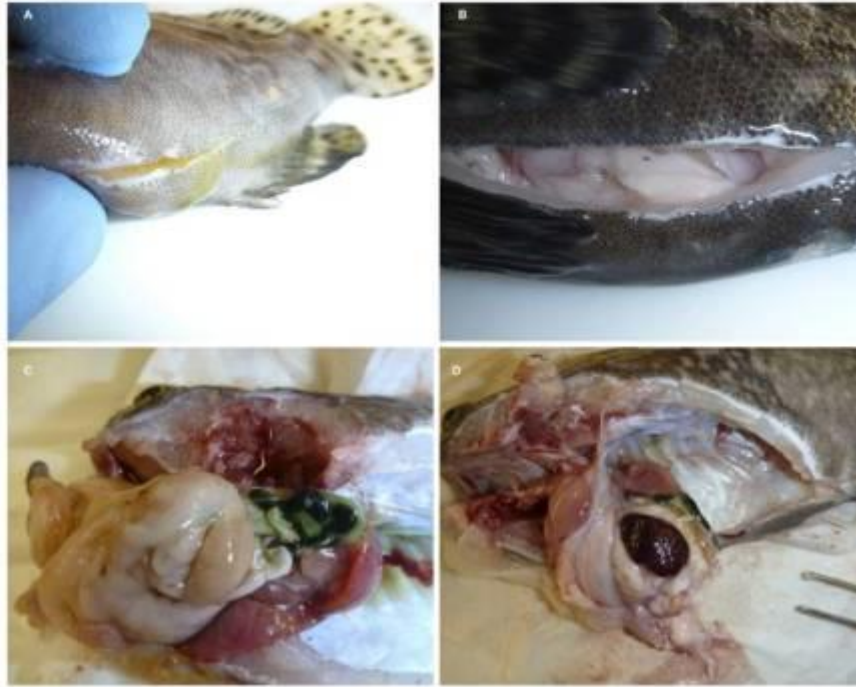

**Figure 13.** Internal clinical signs of streptococcosis in juvenile Queensland grouper. **A**. Fish with ascites (injection challenge trial, high dose,$10^6$ cfu fish$^{-1}$); **B**. normal peritoneal cavity (control fish, note absence of fluid); **C**. full gall bladder (green) within the peritoneal cavity of a fish with an empty stomach; **D**. splenomegaly (immersion challenge trial, high dose $10^6$ cfu ml$^{-1}$).

**Combined laboratory test results (gross clinical signs, bacteriology, PCR and histopathology)**

***Experiment 2: Injection challenge trial***

***Replicate 1***

Overall, all three laboratory test results (PCR, bacteriology and histopathology), for all pooled organ samples from each of 34 fish tested, were in 100% agreement, and were positive for *S. agalactiae*. Combined laboratory test results for replicate 1, confirmed that all juvenile grouper, inoculated with different doses of *S. agalactiae*, (ranging from $10^3$ to $10^8$ cfu ml$^{-1}$), become clinically infected with *S. agalactiae,* regardless of the dose rate of inoculum used.

These results indicate that the *S. agalactiae* bacterial strain QMA0825, originally isolated from the kidney of a wild Queensland grouper, *E. lanceolatus*, which died in Townsville in 2010, is highly virulent to juvenile grouper, resulting in overt clinical signs and pathology indicative of streptococcosis in fish, and resulting in death.

Individual PCR results for the injection trial (replicate 1) were in nearly 100% concordance with pathology results, with all 34 fish positive by pathology, also testing positive by PCR, for all organs tested (brain, head kidney and spleen) (Appendix 3). The exception was a

single kidney from one fish that tested negative by PCR, and two organs that were missing (so could not be analysed by PCR).

Individual bacteriology results for the injection trial (with the exception of six individual organs from six fish that tested negative) were mostly in agreement with PCR and histopathology (Appendix 3). 100% of all selected organs tested by histopathology examination of Gram Glynn special stains, were positive for *S. agalactiae*. 30 of 36 organs (83%) tested by bacteriology were positively identified as *S. agalactiae* (Appendix 3). All negative control fish (housed in a separate system), tested by PCR, bacteriology and histopathology were all negative for *S. agalactiae* (Appendix 3), and both individual and pooled organ results were in 100% agreement.

***Replicate 2***

Overall, all three laboratory test results (PCR, bacteriology and histopathology), for all pooled organ samples from each of 33 fish tested, were in 100% agreement, and were positive for *S. agalactiae*. Combined laboratory test results for replicate 2, indicated that all 33 juvenile grouper inoculated with different doses of *S. agalactiae*, (ranging from $10^3$ to $10^8$ cfu/ml), became infected with *S. agalactiae,* regardless of the dose rate of inoculum used (Appendix 4).

All three laboratory test results (pooled organ samples) were in agreement. These results confirm the results of replicate one that the *S. agalactiae* bacterial strain QMA0825, is highly virulent to juvenile grouper, resulting in clinical signs of streptococcosis and death.

Individual organ results for the injection challenge trial (replicate 2) were nearly in 100%, concordance. 92 of 92 (100%) of the selected organs (brain, head kidney and spleen) tested by PCR, were positive for *S. agalactiae*. 90 of 92 (98%) of selected organs tested by histopathology examination of Gram Glynn special stains were positive for *S. agalactiae*. 32 of 36 organs (89%) tested by bacteriology were positively identified as *S. agalactiae* (Appendix 4). Two fish survived the experiment, one was moribund, but the other was not. Both fish tested positive by PCR and histology. This suggests these fish may have been sub-clinical carriers capable of mounting a successful innate immune response.

**Conclusion**

Overall, combined laboratory test results (PCR, bacteriology and histopathology), for the injection challenge trial (both replicates 1 and 2), indicate that juvenile grouper, inoculated with different doses of *S. agalactiae*, (ranging from $10^3$ to $10^8$ cfu/ml), become clinically infected, regardless of the dose used, show clinical signs of streptococcosis and die within three to four days (except for two fish in replicate 2).

These results fulfil Koch's postulates, and indicate that *S. agalactiae* (bacterial strain QMA0825, originally isolated from the kidney of a wild Qld grouper *E. lanceolatus* that died in Townsville in 2010), is highly virulent to juvenile grouper, resulting in overt clinical signs and death within three to four days.

Two fish from replicate 2, that received a low dose treatment ($10^3$ cfu/ml), survived the 15 days of the experiment; one fish was moribund with clinical signs of disease (bilateral exophthalmia and a congested anus), the other fish showed no clinical signs of disease (Appendix 4). Both fish tested positive by PCR and histology. These fish may have been sub-clinical carriers, effectively mounting an innate immune response and surviving.

Alternatively, these fish may have not received a full dose during injection (eg. from human error during injection) and perhaps developed a delayed infection.

**Experiment 3: Immersion challenge trial**

***Replicate 1***
Laboratory results showed that juvenile grouper can become infected with *S. agalactiae* by immersion in seawater, inoculated with $10^3$, $10^4$ or $10^5$ cfu $ml^{-1}$ *S. agalactiae*. Not all test results were in agreement. *S. agalactiae* was not isolated from fish tested from all three treatment groups (low, medium or high doses), but was isolated from positive control fish. In contrast, PCR analysis, done on whole tissues, showed 3 of 4 fish in the low dose, 1 of 4 fish in the medium dose, and all four fish from the high dose treatment groups tested positive (Appendix 5). Histopathology analysis similarly showed that some fish, from all 3 treatment groups were infected with *S. agalactiae* and had mild pathology indicative of streptococcosis. This was interesting, since all fish (from all three treatment groups) survived the entire 15 days of the experiment, showing no overt clinical signs of disease, except for one fish with red skin lesions, that was from the high dose treatment group.

All positive control fish, tested positive for *S. agalactiae*, by PCR, bacteriology and histopathology, and exhibited clinical signs of infection, including exophthalmia, skin lesions and erratic swimming. All fish from the sentinel control and negative controls groups tested negative for *S. agalactiae* by bacteriology, PCR and histopathology analysis (Appendix 5).

***Replicate 2***
Results for replicate 2, similarly indicated juvenile grouper can become infected with *S. agalactiae,* by immersion of fish in seawater, inoculated with $10^3$,$10^4$ or $10^5$ cfu/ml of *S. agalactiae*. Not all test results were in agreement. *S. agalactiae* was isolated from both fish tested from high dose treatment group, both fish tested from the medium treatment, but not from fish tested from the low dose, or sentinel control groups. Fish from which *S. agalactiae* was isolated also showed clinical signs of disease. In contrast, PCR analysis, done on whole tissues, showed three of four fish in the high dose, four of four fish in the medium dose, and three of four fish from the low dose treatment groups, and all four sentinel control fish, tested positive (Appendix 6).

PCR results were not always in concordance with histopathology results. For example, in replicate 2; 15/16 fish tested positive for *S. agalactiae* by PCR, but only 13/16 fish tested positive by pathology (using H&E & Gram Glynn special stains). It must also be noted that since PCR is detecting bacterial DNA, it is possible that fish that test positive may have overcome the bacterial infection, and may in fact represent a sub-clinical carrier status. Alternatively is also possible fish were in the early stages of bacterial infection when the experiment was terminated and all fish were euthanased.

Surviving, non-clinical fish tested both positive and negative by PCR. This also suggests that it is possible some of these fish were only just becoming infected, (when the experiment was terminated on day 15). It is possible these fish were only just beginning to develop clinical signs, or, that these fish were survivors and were subclinical carriers.

Individual organ results for each fish (for both replicates 1 & 2) showed that not all three diagnostic test results (for each organ) were in agreement (Appendix 6). Consultation with a senior DAFF statistician indicated there was insufficient data for meaningful statistical

comparison to be done on individual organ test results. False negative and false positive results can arise from sampling error, human error, (eg. sample mix-up, test methodology), non-specific factors etc. and were taken into consideration when drawing final conclusions from these results.

***Conclusion***

Overall, combined laboratory test results, from the immersion challenge trial (replicates 1 and 2), indicate that juvenile grouper can become infected with *S. agalactiae* after immersion for one hour in seawater inoculated with three different doses ($10^3$,$10^4$ or $10^5$ cfu $ml^{-1}$) of the bacterium. These results show that the bacterial strain QMA0825 (that killed a wild adult Queensland grouper in Townsville), is highly virulent to healthy naive juvenile grouper, and that *S. agalactiae* can infect juvenile Qld grouper, via infected water.

Results also show that *S. agalactiae* can move between tanks, ie. via recirculating infected water, and in this way fish in another tank can become infected, (within the same recirculating system), despite the physical separation of tanks, and despite the water from each tank being filtered through to a biological filtration system, with an added protein skimmer. Note that water used in this system was not UV sterilised, or ozone treated.

**Histopathology interpretation of juvenile Qld grouper examined from experiment 2, the injection challenge trial**

***Replicate 1***

Histopathology examination of 34 juvenile Qld grouper from replicate 1 of the injection challenge trial, showed all 34 fish were infected with *S. agalactiae*. All 34 fish examined had evidence of streptococcosis, with a systemic bacterial infection and multi-organ involvement. Fish from all six treatment doses ($10^3$, $10^4$, $10^5$, $10^6$, $10^7$ and $10^8$ cfu $fish^{-1}$) had evidence of bacterial septicaemia (Appendix 3). A mixed, but predominantly granulomatous inflammatory response, consisting of aggregations of both mononuclear inflammatory cells, and macrophages containing intracellular, Gram-Glynn-positive cocci, was visible in all organs and tissues examined, indicating widespread organ involvement. The brain, eye, gills, heart, head kidney, caudal kidney, spleen, liver, pancreas, stomach, intestine, visceral and parietal peritoneum, swim bladder, skin and muscle were all affected. Pathological diagnoses made included meningitis, ophthalmitis, exophthalmos, keratitis, choroiditis, branchiitis, splenitis, interstitial nephritis, hepatitis, gastritis, enteritis, pancreatitis, peritonitis, myositis and ulcerative and hyperaemic dermatitis. Pathologic lesions observed in each of the 34 fish are briefly summarised in Appendix 3.

Negative control fish (housed in a separate recirculation system, located away from experimental fish) displayed some very minor pathology, but it was not indicative of infection with *S. agalactiae*. No Gram-positive cocci bacteria were detected from examination of special stains (Gram Glynn) done on all organs and tissues, from these fish. One fish had a bacterial infection that was confined to the liver. There were multiple bacterial granulomas in the liver, the granulomas containing a central core of Gram-negative rod-shaped bacteria. There was a very mild pericarditis consisting of a few infiltrates of mononuclear inflammatory cells, and an abnormal swim bladder. This fish tested negative for *S. agalactiae* by both bacteriology and PCR. Two fish had very mild steatitis of adipose tissues surrounding pancreatic tissue in the abdominal cavity.

A general summary of the main histopathological lesions observed, from all fish, from all injection challenge trial treatment groups, is outlined below.

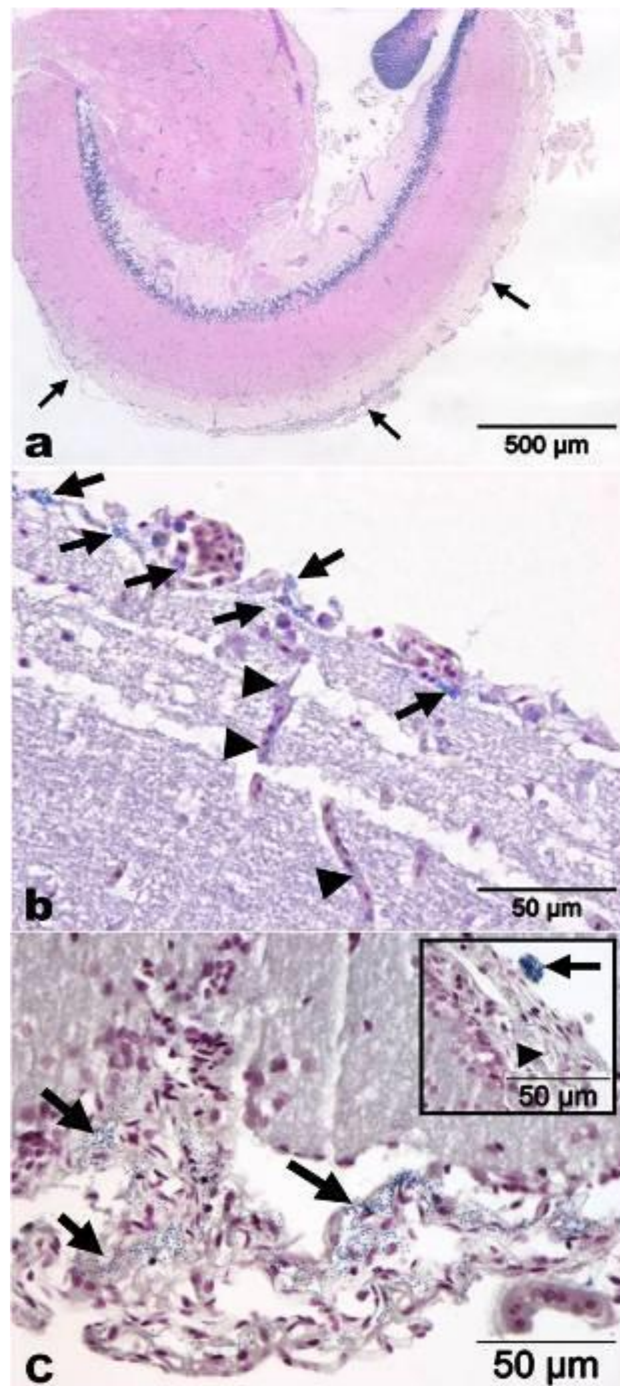


**Figure 14.** Photomicrographs of histological sections from the brain of a juvenile Queensland grouper (Injection trial, treatment 2) infected with *S. agalactiae***.** (**a**) The meninges are thickened with a mixed inflammatory infiltrate (arrows) (X40) H&E stain; (**b**) Higher power view of meninges showing *S. agalactiae* bacteria (blue-staining) in macrophages in dilated meningeal blood vessels (arrows) and in blood capillaries (arrowheads) (X400) Gram Glynn; (**c**) *S. agalactiae* bacteria in the meningeal blood vessels. Inset shows Gram-Glynn-positive cocci inside a macrophage in the meninges (arrow) and in blood capillaries (arrowhead) (X400) Gram Glynn.

The meninges of the brain were thickened with a mixed inflammatory infiltrate (Fig. 14a). Colonies of Gram-Glynn-positive cocci were detected in the meningeal blood vessels and in blood capillaries in the white matter (Fig 14b). Gram-Glynn-positive cocci were also detected in circulating macrophages in the third ventricle, and in the meninges (Fig. 14c, Inset). Occasionally there were multiple, large granulomas occupying the third ventricle.

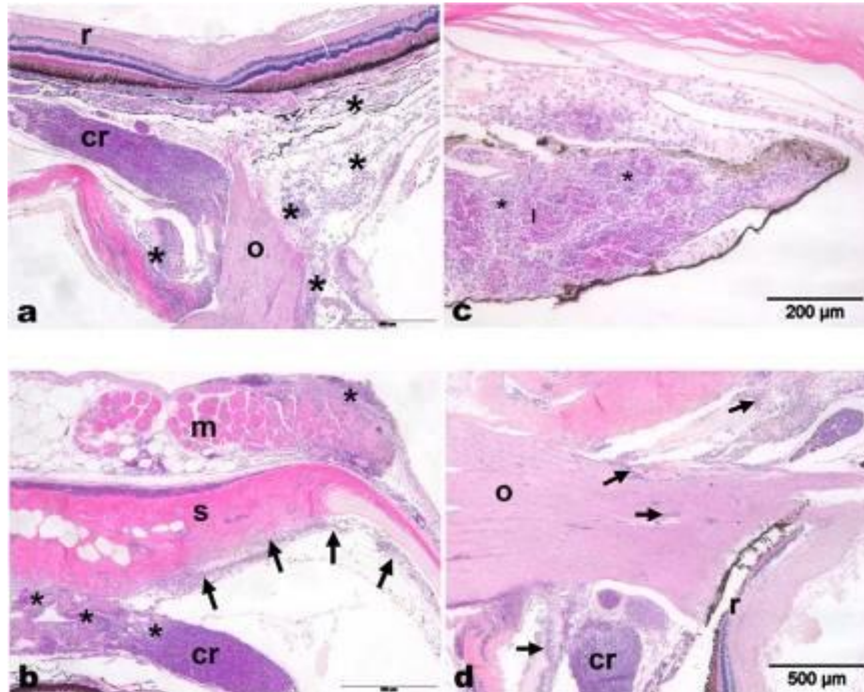


**Figure 15.** Photomicrographs of histological sections from the eye of juvenile grouper from the injection challenge trial (replicate 1) infected with *S. agalactiae* stained with H&E. (**a**) Ophthalmitis, with infiltrates of granulomatous inflammatory cells in the choroid rete (cr), choroid space (*), and adjacent to the optic nerve (o) (X40) scalebar 500 µm; (**b**) Inflammation of the sclera (s) (arrows), choroid rete (cr) (*) and periocular skeletal muscles (m) (*) with mixed and granulomatous inflammatory cells (X40) scalebar 500 µm; (**c**) Aggregations of inflammatory cells (*) in the iris (X200); (**d**) pockets of inflammatory cells within the optic nerve (o) and in the choroid space (arrows) (X40).

The eyes of many fish examined had marked bilateral or unilateral exophthalmia, with varying degrees of inflammation, ranging from moderate to severe (Fig. 15a). The choroid was the main part of the eye affected (choroid rete, choroid space and lamina choroid capillaris) (Fig. 15b, d). The iris, sclera, cornea, peri-ocular muscles and connective tissues were also affected, and sometimes the falciform process (Fig. 15b, c & 16e, f). Colonies of Gram-Glynn-positive cocci were detected free within the choroid space or anterior chamber, or within macrophages in most parts of the eye (Figs 16a, b, c, d, e, f). Gram-Glynn-positive cocci were detected in the anterior chamber of the eye in some fish (Fig. 16c, d). Occasional fish had keratitis, with pockets of inflammatory cells visible in the cornea, mainly in the limbic region, extending bilaterally towards the frontal cornea and the sclera (Fig. 16e). The cornea was most often thickened and oedematous at the limbic region, with increased space between adjacent stromal layers, and occasional separation of Descemet’s membrane from the substantia propria.

The gills showed a generalised branchitiis, with multifocal, proliferative hyperplasia of gill lamellar epithelium and fusion of adjacent gill lamellae (Fig.17a, b). The tips of the gill filaments were often thickened with a granulomatous inflammatory infiltrate (Fig. 17a). In

most fish examined, there was a low grade, generalised inflammation along the length of a few, but not all, gill filaments were affected (Fig. 17d). Branchial blood vessels of the gill lamellae and gill filaments were dilated and congested, containing Gram-Glynn-positive cocci either in colonies or within circulating erythrocytes or macrophages (Fig. 17c, e & f). Macrophages in the gill lamellae, or sloughed, containing Gram-Glynn-positive cocci, were detected between adjacent gill lamellae, indicating resident or wandering macrophages within the gills may play a role in engulfing and ridding the fish of bacteria.

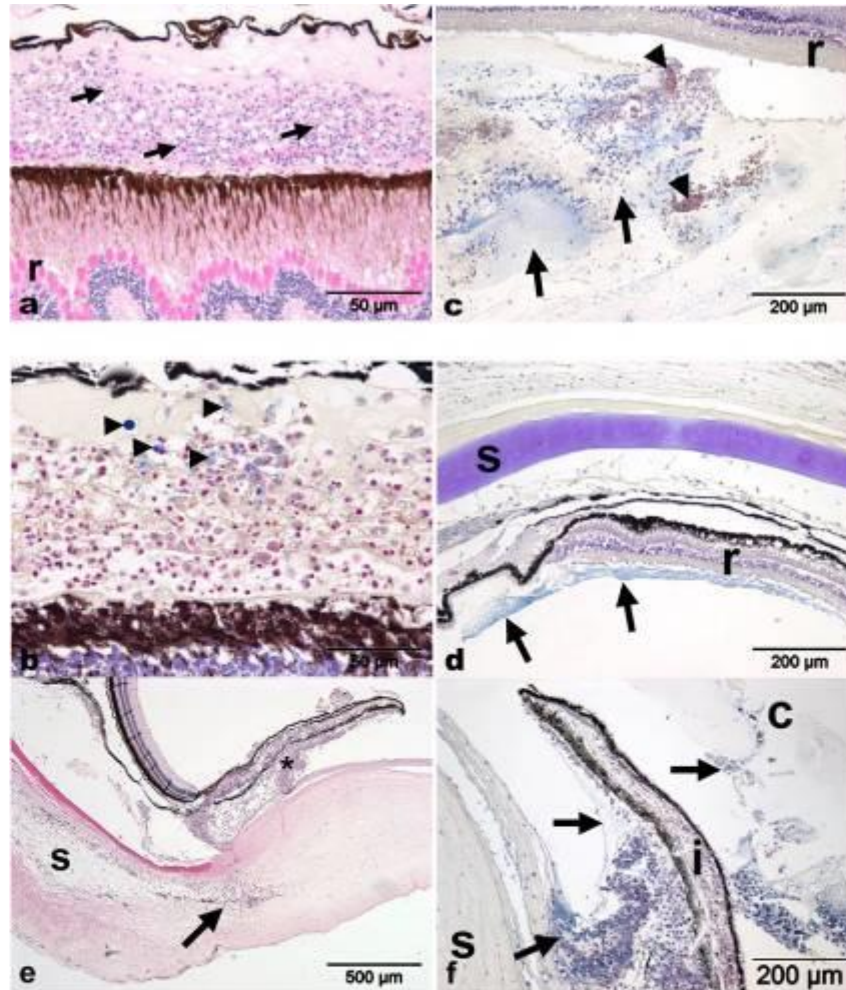


**Figure 16.** Micrographs of histological sections of the eyes from juvenile grouper from the injection challenge trial (replicate 1) infected with *S. agalactiae* (**a**) Inflammation of the choroid capillaris (arrows). r = retina (X400) H&E stain; (**b**) *S. agalactiae* in the choroid capillaris (blue-staining, arrowheads) (X100) H&E stain; (**c**) Haemorrhage (arrowheads) and mixed inflammatory infiltrate, with *S. agalactiae* bacteria in the anterior chamber of the eye (arrows) r = retina (X200) Gram-Glynn stain; (**d**) *S. agalactiae* in the anterior chamber of the eye (arrows) r = retina, s = sclera Gram-Glynn stain. (X200) Gram-Glynn stain; (**e**) eye with keratitis and iritis. Note infiltrates of mixed mononuclear and granulomatous inflammatory cells in the cornea (arrow); scleral (s) connective tissue with oedema and separation of stromal layers, and inflammation (arrow); and inflammation of the ciliary cleft (*) (X40) H&E stain; (**f**) Inflammation of the ventral ciliary cleft (arrows), showing aggregations of macrophages containing Gram-Glynn-positive cocci, i = iris, (X100) Gram-Glynn stain.

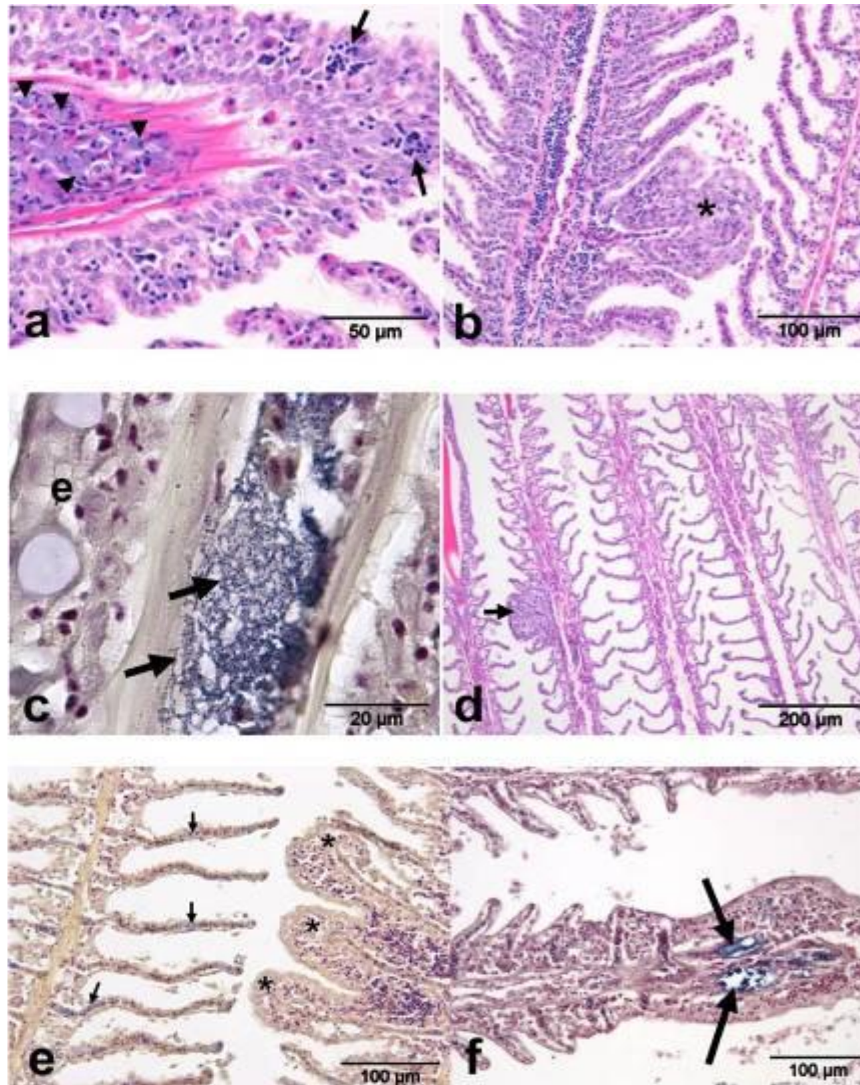


**Figure 17.** Micrographs of histological sections of gills from juvenile grouper from the injection challenge trial (replicate 1) infected with *S. agalactiae.* (**a**) The tips of the gill filaments are thickened with inflammatory cells (arrows). Note macrophages containing *S. agalactiae* cocci-shaped bacteria in blood vessel lumen (arrowheads). H&E (X400); (**b**) Branchitis with infiltrating mononuclear inflammatory cells in blood vessel lumen, and foci of granulomatous inflammation, with fusion of adjacent gill lamellae (*) (X100) H&E; (**c**) Colonies of *S. agalactiae* in blood vessel of gills (arrows). e=epithelium. (X1000) Gram Glynn; (**d**) Low power view of gills showing foci of inflammation (arrow) , synechiae of adjacent gill lamellae (X100) H&E; (**e**) *S. agalactiae* bacteria (blue-staining) in macrophages in gill lamellae (arrows) Gram-Glynn stain (X200); (**f**) Colonies of Gram-Glynn-positive cocci in blood vessel lumen of gills (X200) Gram-Glynn stain.

Histopathological examination of the heart (from all fish) showed a moderate to severe haemorrhagic pericarditis (Fig. 18a). The pericardium of the ventricle and bulbus arteriosus was markedly thickened, compared to the atrium (Fig. 18a, b). The inflammatory infiltrate often extended into the myocardium of the ventricle, or myoelastic tissue of the bulbus arteriosus. There were infiltrates of mixed granulomatous inflammatory cells, mostly macrophages, containing Gram-Glynn-positive cocci, into the pericardium (Fig. 18b). Bacteria were visualised, free in the pericardial blood capillaries, or adhered to the endothelial lining of blood vessels or adhered to erythrocytes (Fig. 18e). In many fish there was swelling of the endothelial macrophages (Fig. 18c). Endothelial macrophages contained Gram-Glynn-positive cocci (Fig. 18d). In a few cases there was endocarditis, with dysplastic

thickening of the atrio-ventricular valve (Fig. 18f) or other heart valves. One fish had an unusual and rare heart lesion consisting of cartilaginous metaplasia of the ventricular myocardium (not shown here).

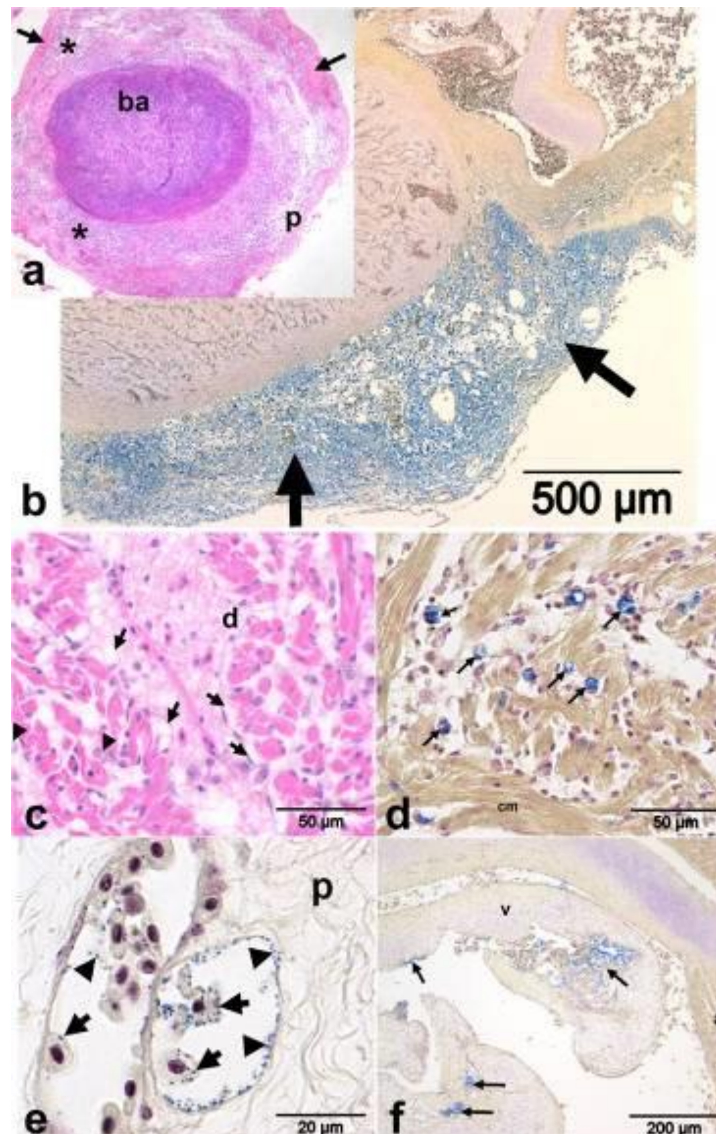


**Figure 18.** Micrographs of histological sections of the heart from juvenile grouper from the injection challenge trial infected with *S. agalactiae* (**a**) The bulbus ateriosus (ba) of the heart with marked pericarditis (p). Note the granulomatous (*) and fibrinous (arrow) inflammatory infiltrate in the pericardium (X40) H&E; (**b**) The pericardium (p) is markedly thickened with granulomatous inflammatory exudate (arrows) (X40) Gram Glynn; (**c**) Swollen, dilated endothelial cells (arrows) of the ventricular myocardium. (**d**) Gram Glynn stain showing endothelial macrophages replete with Gram Glynn positive staining *S. agalactiae* (arrows) and degenerating myocardium (X400) cm=cardiac muscle; (**e**) Colonies of *S. agalactiae* (blue staining, arrows) in the pericardial (p) blood vessels, adhered and within erythrocytes (arrows) and adhered to endothelial lining (arrowheads) (X1000). (**f**) Endocarditis of AV valve (v). Note valve dysplasia and colonies of *S. agalactiae* (blue staining, arrows) adhered to valve (v). a = atria (100X) Gram-Glynn.

The liver had a generalised hepatopathy and hepatitis, with variable glycogen stores in the hepatocytes. Perivascular inflammation was commonly observed with aggregations of macrophages and colonies of Gram-Glynn-positive cocci in the vessel lumens, sometimes with sloughed endothelium (Fig 19a, b). There were aggregations of granulomatous

inflammatory cells adjacent to blood vessels (Fig. 19c). Gram-Glynn-positive cocci were detected in blood vessel lumens (Fig 19d).

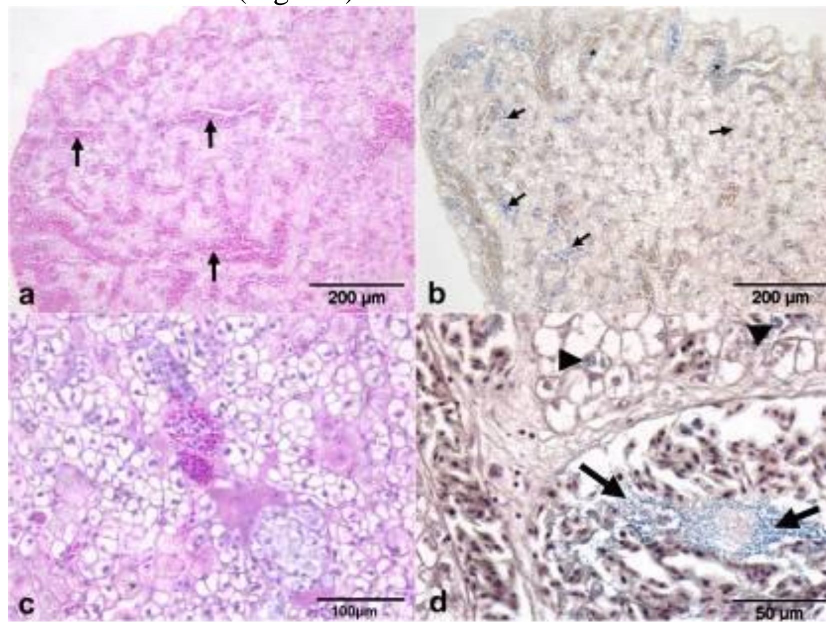


**Figure 19.** Micrographs of histological sections of liver from a juvenile grouper infected with *S. agalactiae* (injection trial, System 1). (**a**) Liver with hepatitis. Note dilated sinusoids (arrows) (X100) H&E; (**b**) Gram Glynn demonstrates numerous Gram-Glynn-positive cocci (blue, arrows) in macrophages among hepatic sinusoids (X100) Gram Glynn; (**c**) Foci of granulomatous inflammation and perivascular inflammation, with variable glycogen storage in hepatocytes (X200) H&E; (**d**) Colonies of *S. agalactiae* bacteria in blood vessel (arrows) and in macrophages (arrowheads) throughout the parenchyma (X400) Gram Glynn.

The head kidney had haemorrhagic nephritis, with scattered, multifocal necrosis of the haematopoietic tissue. Necrotic areas were often circular and confluent, consisting of granulomatous-like areas of inflammation, and haemorrhage, associated with Gram-Glynn-positive cocci (Fig. 20b). There was phlebitis, and rarely, thrombophlebitis (Fig 20a, c & d). The caudal kidney had nephritis and nephropathy. There was scattered, depleted but rarely necrosis, of the haematopoeitic tissue, with a generalised low grade inflammation of the haematopoietic tissue and phlebitis (Fig. 20c). Only rarely was there necrosis and degeneration of tubule epithelium or glomeruli. Gram-Glynn-positive cocci were seen in macrophages among haematopoietic tissue and in blood vessel lumens (Fig 20c, d). The spleen was congested and haemorrhagic, with degeneration and necrosis of ellipsoids, and depletion of the haematopoietic and lymphomyeloid tissue (Fig. 20f). Gram-Glynn-positive *S. agalactiae* were detected in colonies, or within enlarged macrophages throughout the spleen (Fig. 20e, f)

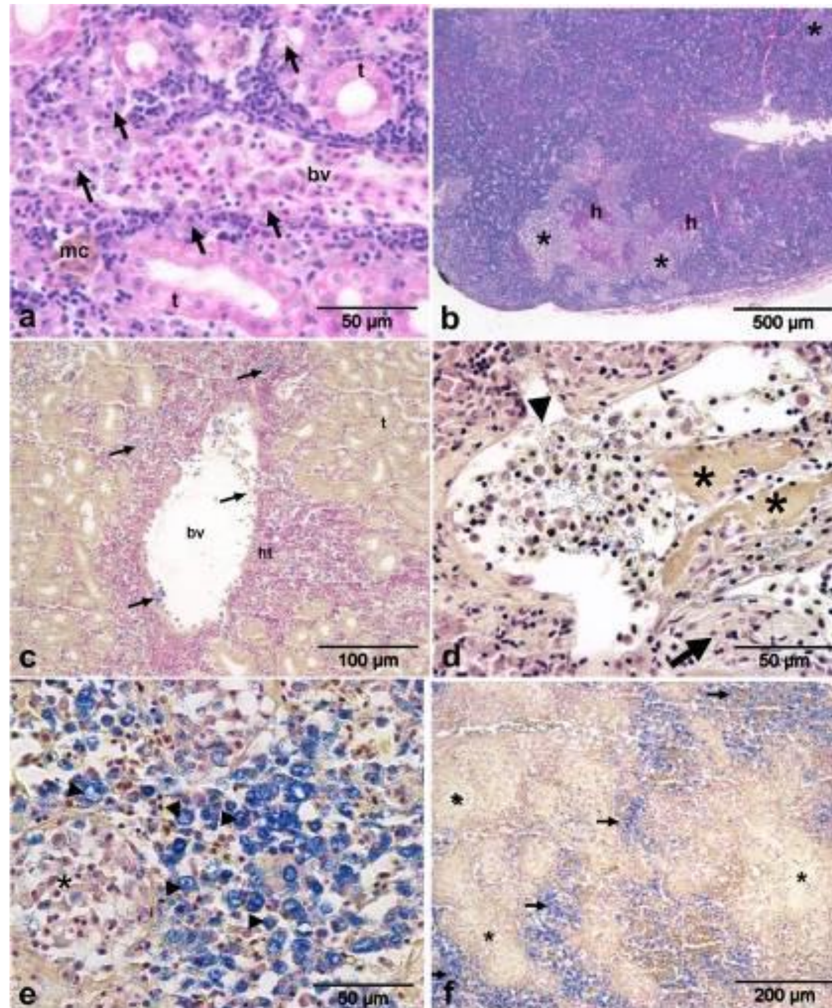


**Figure 20.** Micrographs of histological sections of the head and caudal kidney, and spleen from a juvenile grouper from the injection challenge trial (replicate 1), infected with *S. agalactiae*. (**a**) Caudal kidney with phlebitis, note numerous inflammatory cells (arrows) in blood vessel lumen (bv), mc=melano-macrophage centres. (400X) H&E stain; (**b**) Head kidney with multifocal areas of inflammation (*) and haemorrhage (h) (X40) H&E stain; (**c**) Phlebitis (arrow) with numerous Gram-Glynn-positive (blue) cocci in macrophages among haematopoietic tissue (ht), and in blood vessel (bv) lumens. (X200) Gram-Glynn; (**d**) Thrombophlebitis of the head kidney, note inflammation in the blood vessel lumen, with swollen, necrotic endothelium (arrow), with thrombi (*), and numerous cocci (arrowhead) (X400) Gram-Glynn; (**e**) Colonies of *S. agalactiae* (arrows, blue) are throughout the spleen, mainly surrounding, but also within the ellipsoids (*) (X100); (**f**) Colonies of *S. agalactiae* bacteria (blue, arrowheads) in macrophages in both the white and red pulp (X400) Gram-Glynn.

The stomach showed scattered pycnotic necrosis of the mucosal epithelium, of gastric gland cells (Fig. 21a). Colonies of Gram-Glynn-positive cocci were detected in blood vessel lumens in the lamina propria (Fig. 21c). In the submucosa, there were often multiple aggregations of granulomatous-like inflammatory cells in the loose connective tissue layers or veins (Fig 21b). There was pooling of a proteinacous-like substance in the vessels of the muscularis layers (Fig. 21d).

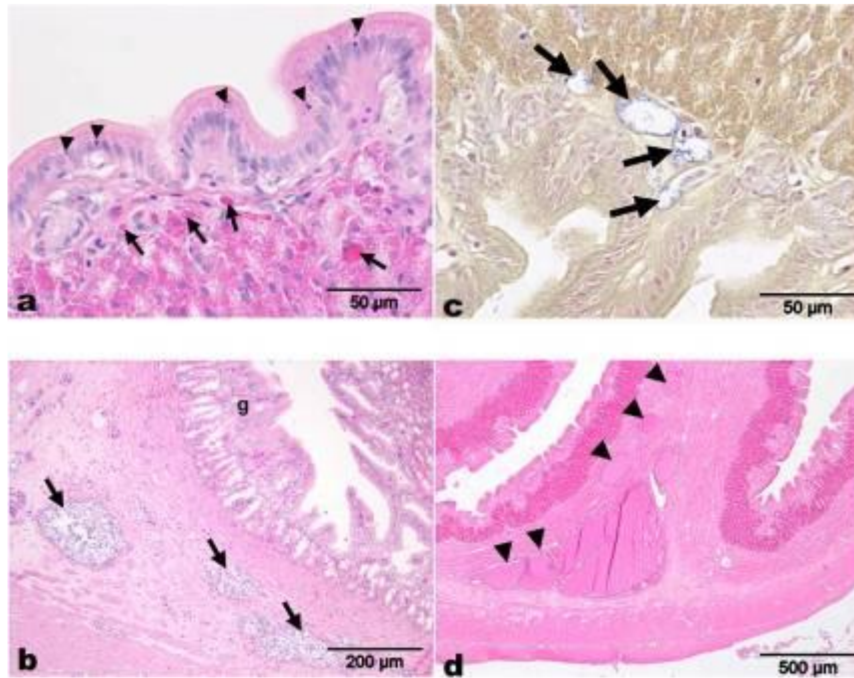


**Figure 21.** Micrographs of histological sections of the stomach from a juvenile grouper infected with *S. agalactiae* from the injection trial. (**a**) stomach showing scattered necrotic cells (arrowheads) in mucosal epithelium and gastric glands (arrows) (X400) stained with H&E; (**b**) submucosa of stomach with aggregations of granulomatous inflammatory cells (arrows) in the veins (X100) stained with H&E; (**c**) colonies of Gram Glynn-positive staining cocci bacteria in vascular network of lamina propria (arrows) (X400) stained with Gram-Glynn; (**d**) pooling of proteinaceous material (arrows) in veins and lymphatic ducts of submucosa (X40) stained with H&E.

There was a marked peritonitis and steatitis in nearly all fish examined. The adipose tissues of the peritoneum were inflamed with infiltrations of macrophages laden with Gram-Glynn-positive cocci, the inflammatory infiltrate often involving the serosa of the intestines, stomach, mesenteric tissues and capsule of the liver (Fig. 22a, b).

There was enteritis in nearly all fish histologically examined, with sloughing of mucosal epithelium into the intestinal lumen, and colonies of Gram-Glynn-positive cocci in the lumen (Fig. 22b). Some fish had haemorrhagic enteritis, with congested blood vessels, sloughing, necrosis and haemorrhage of the mucosal epithelium. The lamina propria of the pyloric caecae was largely dilated, with oedema and colonies of Gram-Glynn-positive cocci & dilated blood vessels (Fig 22a). The serosa was markedly inflamed with a diphtheritic-like membrane made up of granulomatous inflammatory cells, and macrophages containing Gram-Glynn-positive cocci, extending into the peritoneum (Fig. 22c).

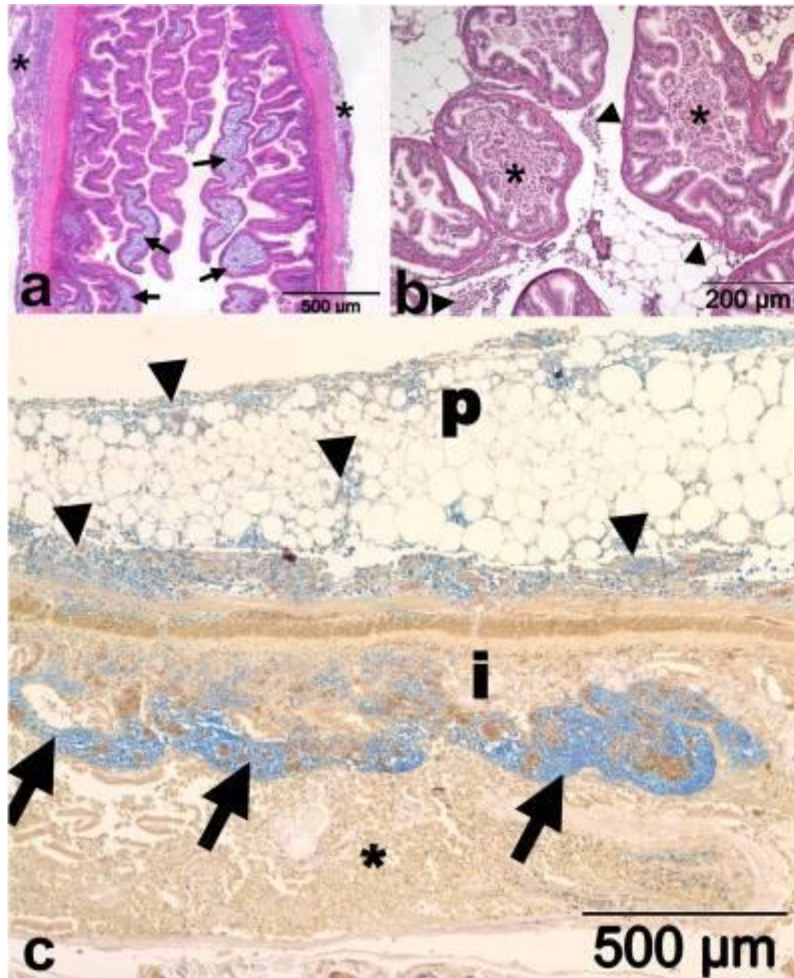


**Figure 22.** Micrographs of histological sections of intestine from a juvenile grouper infected with *S. agalactiae* from the injection trial. (**a**) Intestine with dilated intestinal loops (arrows) containing colonies of coccoid bacteria (X40); (**b**) Pyloric caecea with enteritis (*) and peritonitis (arrowheads) stained with H&E (X100); (**c**) Gram-Glynn stained section of intestine showing enteritis (*), with numerous blue-staining *S. agalactiae* bacteria (arrowheads) in the sloughed necrotic mucosal epithelium (arrows), serosa, and all other parts of intestine, peritoneal adipose tissue (p) and serosa (arrowheads) (X40).

In some fish histologically examined, the skeletal muscle had a few small areas of skeletal muscle myositis and fasciitis. Colonies of Gram-Glynn-positive cocci were detected in the lumen of blood vessels and invading along the fascial planes, epimysium and perimysium between adjacent muscle fibre bundles (Figs 23a, b & c).

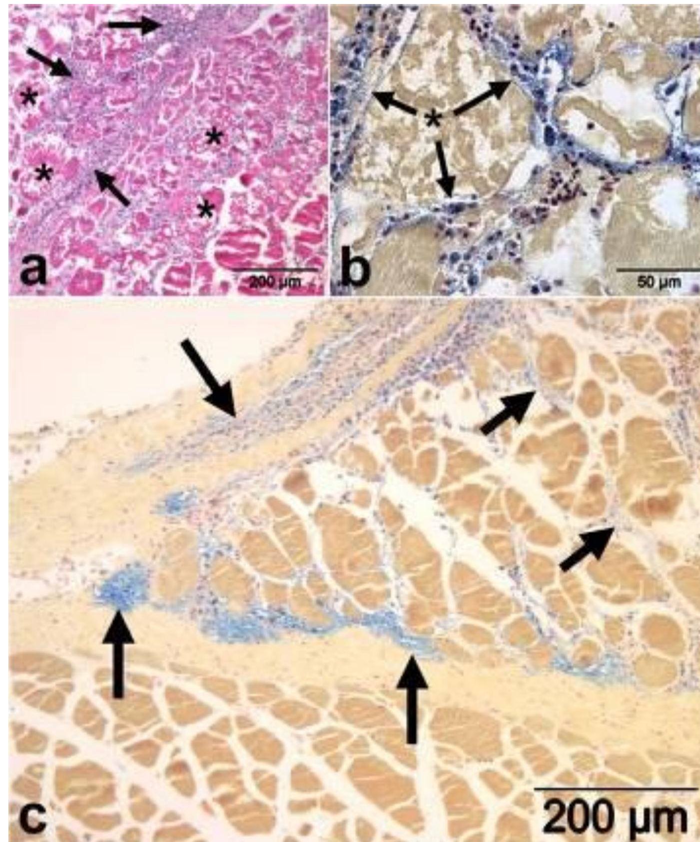


**Figure 23.** Micrographs of histological sections skeletal muscle from a juvenile Qld grouper infected with *S. agalactiae* from the Injection challenge trial (**a**) Skeletal muscle inflamed with a mixed inflammatory infiltrate (arrows) along fascial planes, perimysium and endomysium. Note numerous degenerating skeletal muscle fibres (*) stained with H&E (X100); (**b**) Numerous Gram-Glynn-positive cocci (blue-staining) are invading the endomysium and perimysium. Note the degenerating muscle fibre (*) (X400) stained with Gram-Glynn; (**c**) The fascial connective tissues, perimysium and endomysium of the muscle fasciculi are inflamed, with invading *S. agalactiae* bacteria (blue-staining, arrows) (X100) Gram-Glynn stain.

The skin and fins of many fish histologically examined, had focal areas of oedema, and dermatitis. There was a mixed inflammatory infiltrate, of mononuclear and granulomatous cells, many macrophages containing Gram-Glynn-positive cocci, in the hypodermal connective tissues (Fig. 24a, b).

The swim bladder of several fish was inflamed and haemorrhagic, with a mixed, mononuclear and granulomatous inflammatory infiltrate invading the connective tissues and surrounding the vascular network of the rete (Fig. 24c). Macrophages containing Gram-Glynn-positive cocci were visible in the mixed inflammatory infiltrate (Fig. 24d).

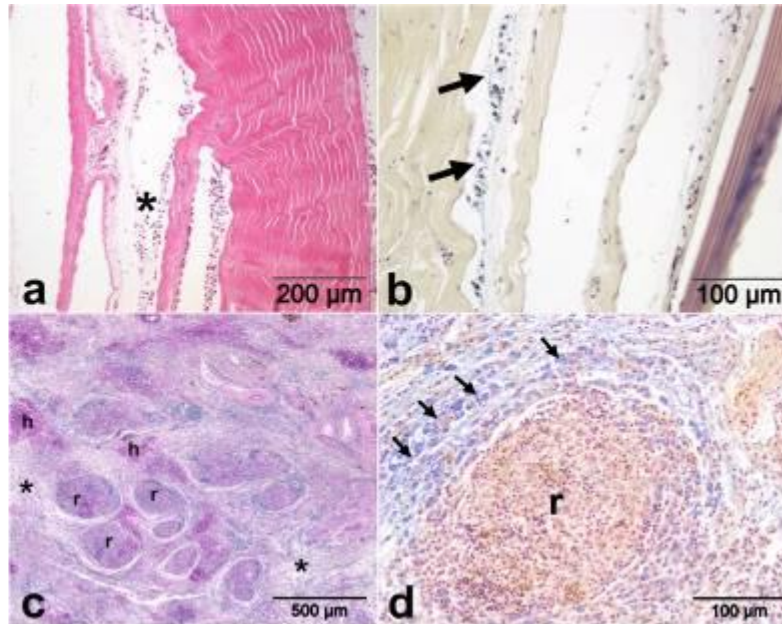


**Figure 24.** Micrographs of histological sections of the skin and swim bladder from a juvenile Qld grouper from the Injection trial infected with *S. agalactiae*. (**a**) Fins, dermis, showing infiltration of inflammatory cells into the hypodermis (*) (X100) H&E stain; (**b**) Numerous Gram-positive *S. agalactiae* cocci (blue-staining) identified by Gram-Glynn stain, seen invading the hypodermis (arrows) (X200); (**c**) swim bladder showing haemorrhage (h) of rete mirabile (r), surrounded by massive infiltrate of mixed granulomatous inflammatory cells (x40) H&E stain; (**d**) Higher power view of rete mirabile (r) showing infiltrating macrophages (arrows) containing blue-staining Gram-Glynn-positive cocci (*S. agalactiae)* (X200).

***Replicate 2***

Histopathology analysis of 33 juvenile Queensland grouper examined from replicate 2 of the injection trial, showed 31 of 33 fish examined had evidence of systemic bacterial infection, with multi-organ involvement. 31 fish examined from all treatment doses ($10^3$, $10^4$, $10^5$, $10^6$, $10^7$ and $10^8$ cfu fish$^{-1}$), had strong evidence of bacterial septicaemia. Pathology was similar to that seen in affected fish from replicate 1. Fish had a predominantly granulomatous inflammatory response, consisting of aggregations of macrophages containing Gram-Glynn-positive cocci, present in all organs and tissues, indicating widespread systemic organ involvement. Pathological diagnoses and lesions were similar to those already described for replicate 1.  Data for individual fish are summarised in Appendix 4.

The two remaining fish examined, were from the low dose treatment group ($10^3$ cfu fish$^{-1}$). They were the only survivors of this experiment) and were euthanased at the termination of the experiment on day fifteen. Only one of these fish showed clinical signs of disease. Both fish had mild pathology of various organs and tissues, including a mild branchitiis, mild iritis and keratitis (Fig. 25a, b). There was a very mild pericarditis of both the ventricle and bulbus arteriosus and bacteria were visualised within macrophages in the pericardium (Fig. 25f). The kidney contained circular, confluent foci of granulomatous inflammation (Fig. 25c).

Examination of Gram-Glynn stained sections showed Gram-Glynn-positive cocci were present, but they were only observed in some organs and tissues. The bacteria were intra-cellular in the cytoplasm, of both resident and circulating macrophages, and were never detected free, or in chains or in colonies, in any organ examined. Gram-Glynn-positive cocci were detected in macrophages within the pericardium of the heart (Fig. 25f), in the blood

vessel lumens of the head kidney, and in macrophages throughout the white and red pulp of the spleen and ellipsoids (Fig. 25e). In the brain, Gram-Glynn-positive cocci were detected in macrophages, in blood capillaries and in the third ventricle of the brain (Fig. 25d). Gram-Glynn-positive cocci were also detected in sloughed macrophages, between adjacent gill lamellae.

It is possible these two surviving fish represent carriers of the bacteria, or that they were early on in the infection process, since one of these fish showed clinical signs of disease, and the other did not. It is not possible to determine whether both these fish would have survived longer or not (as carriers), or whether the other fish would have developed clinical signs, as the experiment was terminated and all fish were euthanased according to the University of Queensland Animal Ethics Permit.

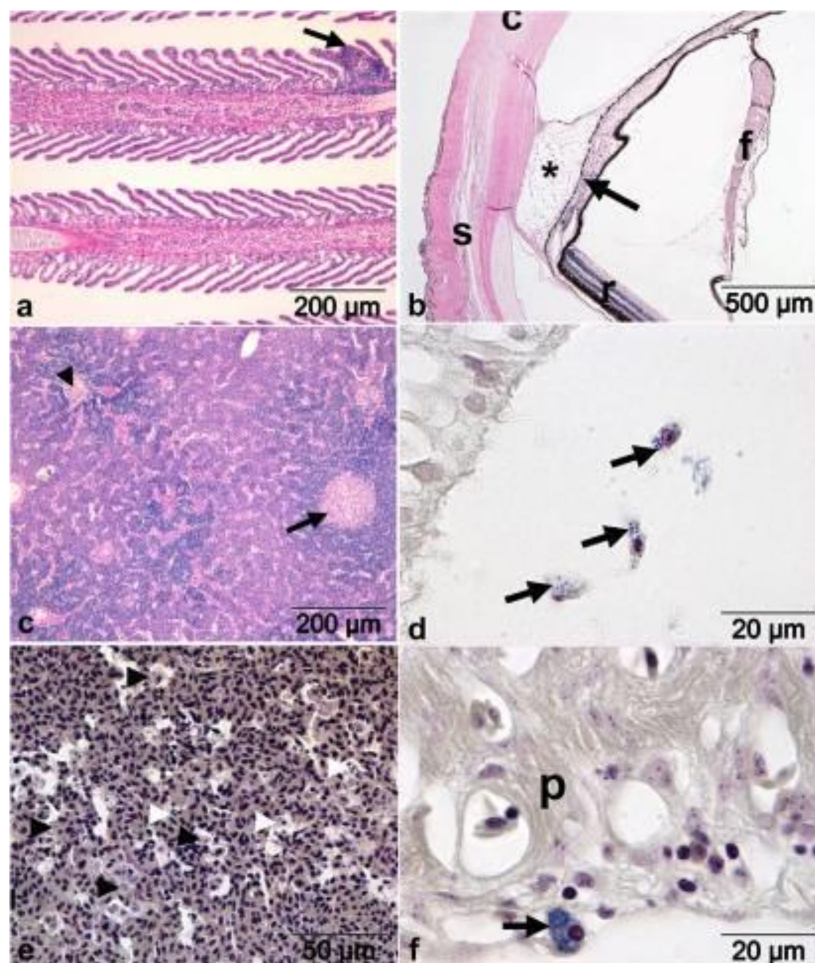


**Figure 25.** Micrographs of histological sections of organs from a surviving juvenile Queensland grouper from the Injection challenge trial (treatment 1, system 2). (**a**) Gills with foci of granulomatous inflammation (arrow) (X100) stained with H&E; (**b**) Eye. Note oedema (*) of ciliary cleft, mild keratitis at limbic regions of sclera (s) extending into cornea (c) and inflammation of the iris (arrow) and falciform process (f) (X100) stained with H&E; (**c**) head kidney with foci of granulomatous inflammation (arrows) (X100) stained with H&E; (**d**) The third ventricle of the brain showing intracellular, Gram-Glynn-positive cocci in circulating macrophages in the CSF (arrows). (X1000). (**e**) Spleen with necrotic cells (white arrowheads) and macrophages containing bacteria (dark arrowheads) (X400) stained with gram Glynn; (**f**) High power view of the pericardium of the heart showing intracellular, Gram-Glynn-positive cocci in a macrophage (arrow). (X1000) stained with Gram-Glynn.

**Histopathology interpretation of juvenile Queensland grouper examined from Experiment 3, the immersion challenge trial**

***Replicate 1***

Juvenile Qld grouper examined from the immersion challenge, (replicate 1), subjected to low ($10^4$ cfu fish$^{-1}$), medium ($10^5$ cfu fish$^{-1}$), or high doses ($10^6$ cfu fish$^{-1}$), of *S. agalactiae,* showed mainly a mononuclear, and rarely a granulomatous, inflammatory response in various organs and tissues, indicating fish were responding to the presence of a bacterial pathogen. The inflammatory response varied in severity from mild to moderate to severe, between fish, within and between different treatment groups.

Fish in all three treatment groups, had Gram-Glynn-positive cocci, visible in various organs and tissues, including the brain, eyes, gills, skin, intestine, peritoneum, spleen and stomach, from histological examiantion. Gram-Glynn-positive cocci, were also detected in sloughed macrophages between adjacent gill lamellae (fish 1, 3 & 4 from low dose; fish 3 from high dose), or in macrophages sloughed from the intestinal epithelium (fish 1 from low dose); or shed from the epithelial surface of the skin (fish 3 from high dose) (Figs 27a, b, c, d & e).

In the low dose treatment group, all four fish histologically examined showed evidence of an inflammatory response, with Gram-Glynn-positive cocci rarely detected, and when present, only in macrophages (Figs 26 & 27). Two fish had a very mild choroiditis (Fig. 27a), with a few Gram-Glynn-positive cocci in macrophages, in the choroid space of the eye. Gram-Glynn-positive cocci were also detected in peritoneal macrophages adjacent to pancreatic tissue (Fig. 27e). Two fish had a marked dermatitis of the fins, with oedema in the stratum spongiosa, marked inflammatory infiltrate, dilated blood vessels, and Gram-Glynn-positive cocci among areas of inflammation and necrotic cells (Fig. 26c & 27c). One fish had a mild branchitiis, with a few Gram-Glynn-positive cocci in macrophages, in focal granulomatous-like lesions in a few gill filaments (Fig. 26b). Two fish had a mild pericarditis with perivascular inflammation of coronary blood vessels in the pericardium of the atrium and ventricle (Fig. 26d). Gram-Glynn-positive cocci were detected within macrophages (Fig. 27f). Two fish also had myocarditis, with multifocal aggregations of mononuclear inflammatory cells in the myoelastic tissue of the bulbus arteriosus. All four fish had mild cholangiohepatitis with perivascular mononuclear inflammation, and infiltrates of mononuclear cells into the parenchyma (Fig. 26f), and rarely, melano-macrophages. One fish had nephritis, with granuloma-like lesions in the interstitial tissue of the kidney (Fig. 26e). Two fish had a necrotizing, granulomatous dermatitis and myositis, with a granulomatous and mononuclear inflammatory infiltrate in the stratum spongiosa, surrounded by necrotic and degenerating muscle fibres, dilated blood vessels, and multiple, large melano-macrophage centres. These changes were associated with Gram-Glynn-positive cocci in macrophages and free colonies of bacteria.

In the medium dose treatment group, all four fish histologically examined had mild branchitis. One fish had choroiditis, iritis (Fig. 28a) and scleritis, with a mixed, monocuclear and granulomatous inflammatory infiltrate mainly in the choroid rete and choroid capillaris. Bacteria were detected, variously, in two fish; in the spleen, head kidney, brain, skin (subdermal connective tissue), caudal kidney (haematopoietic tissue), stomach (submucosa) or in macrophages at tips of gill lamellae. Fish had, variously, nephritis (caudal kidney), splenitis (Fig. 28e), haemorrhagic enteritis (Fig. 28b), mild pericarditis (as described earlier), myocarditis, or skeletal muscle myositis and fasciitis (Fig. 28f). One fish had endocarditis,

with aggregations of mononuclear inflammatory cells adhered to the proximal valve of the bulbus arteriosus and atrio-ventricular valve (Fig. 28c).

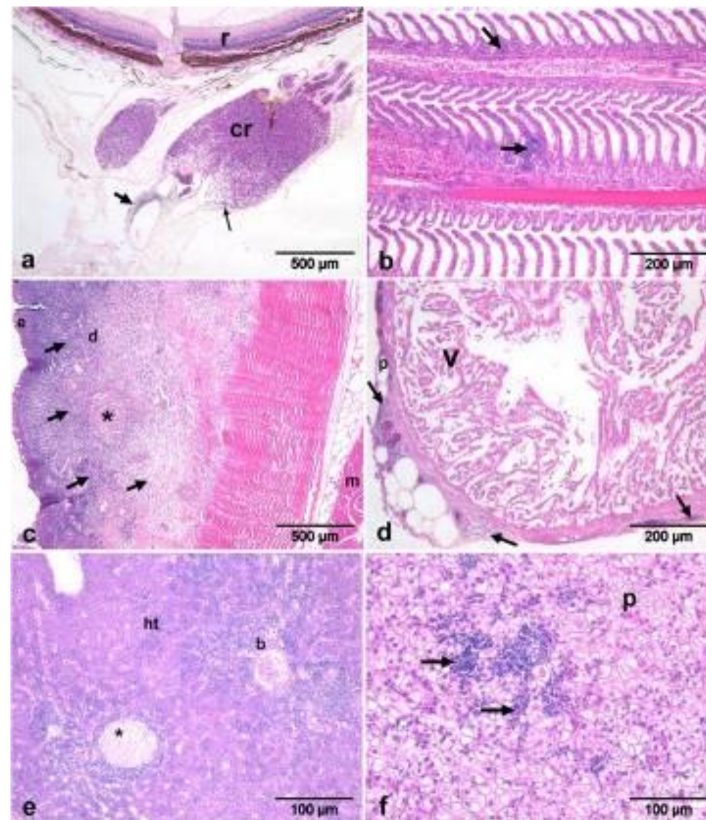


**Figure 26.** Micrographs of histological sections of the eye, gills, skin, heart, kidney and liver from juvenile Queensland grouper from the Immersion challenge trial (low dose, replicate 1) stained with H&E**;** (**a**) eyes with inflammation of choroid rete (cr, thick arrow) (X40); (**b**) gills showing multiple inflammatory lesions (arrows) (X100); (**c**) skin with marked dematitis note inflammatory infiltrate (arrows), granuloma-like lesions (*) (e=epidermis, d=dermis, m=muscle, (X40); (**d**) heart with mild pericarditis (arrows), v=ventricle, p=pericardium (X100); (**e**) head kidney showing granuloma-like lesions (*) among haematopoeitic tissue (ht), b=blood vessel (X100); (**f**) liver with mononuclear inflammatory infiltrate (arrows). p=parenchyma (X200).

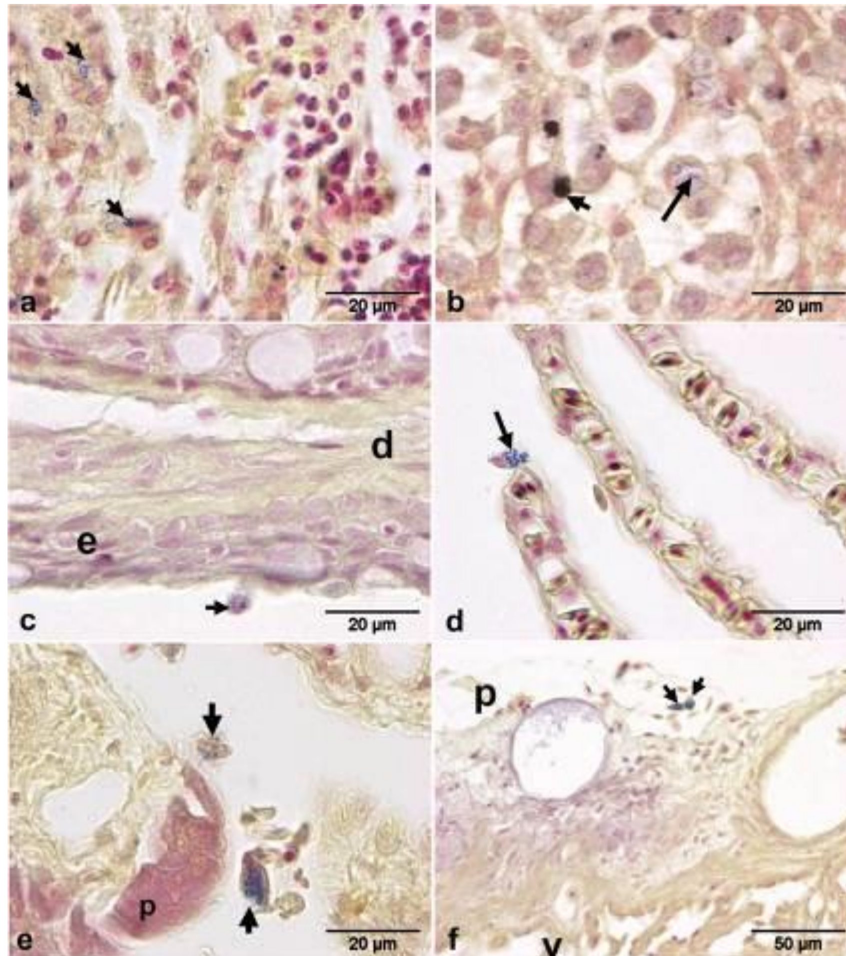


**Figure 27.** Micrographs of histological sections of the skin, gills, peritoneum and heart from juvenile Qld grouper from the Immersion challenge trial (low dose, replicate 1) stained with Gram Glynn special stain; (**a**) epidermis of skin showing inflammation, and multiple macrophages laden with Gram-Glynn positive *S. agalactiae* bacteria (arrows) (X400); (**b**) higher power shot of (a) showing bacteria in a macrophage (long arrow) and necrotic cell (short arrow) (X1000); (**c**) skin epidermis (e) with a single sloughed macrophage (arrow), containing Gram-Glynn-positive cocci, (X100); (**d**) gill lamellae with a sloughed macrophage containing Gram-Glynn-positive cocci (arrow) (X1000); (**e**) peritoneal macrophages laden with Gram-Glynn-positive cocci (arrows) near pancreatic tissue (p) (X1000); (**f**) pericardium (p) of heart with macrophages full of Gram-Glynn-positive cocci (arrows) (X400) v=ventricle.

Fish from the high dose treatment group had a predominantly mixed inflammatory response, with both a monocuclear and granulomatous inflammatory infiltrate. All four fish had mild branchiitis, mild meningitis, mild choroiditis (choroid rete, choroid space and choroid capillaris), and iritis. Two fish had a marked, severe dermatitis, with oedema and haemorrhage of the fins, with a mononuclear cell infiltration into the dermal connective tissue. Gram Glynn-positive cocci were seen in sloughed cells on the surface of the skin or in macrophages deeper in the basal cell layers of the epidermis, associated with large melano-macrophages and necrotic cells. Three fish had changes in the heart and liver (as outlined above). All four fish had splenic congestion.

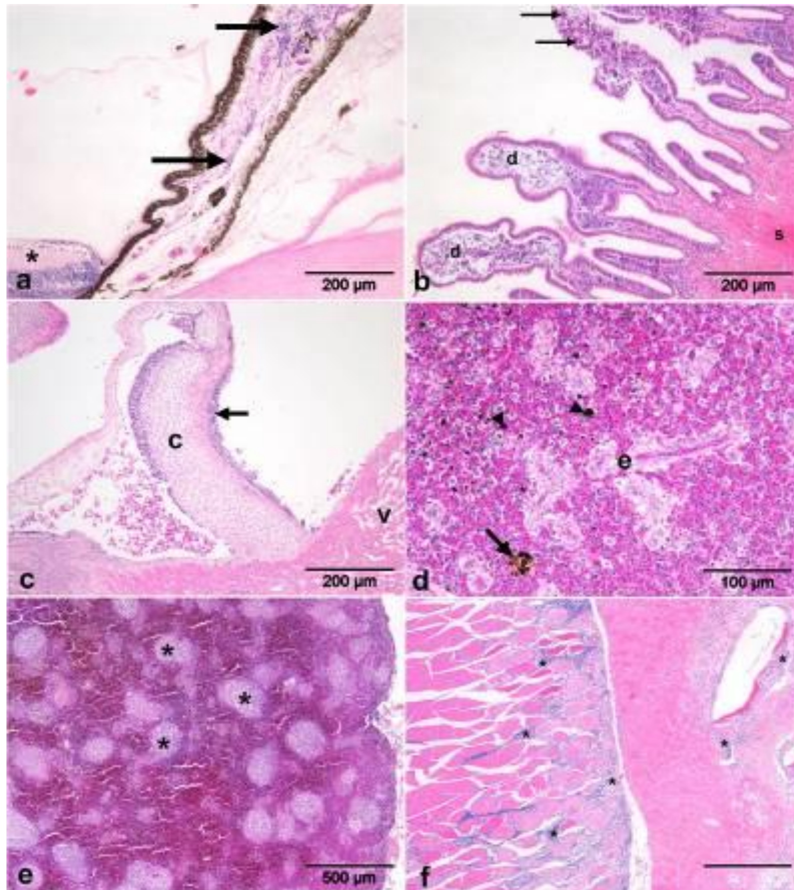


**Figure 28.** Micrographs of histological sections of the eye, intestine, heart, spleen, and skeletal muscle from juvenile Queensland grouper from the immersion challenge trial (medium dose, replicate 1) stained with H&E**;** (**a**) Iris with inflammation (arrow) (X100); (**b**) intestine with dilated intestinal loops (**d**) containing bacteria and haemorrhage (long arrows) (X100); (**c**) atrio-ventricular valve (**c**) with mononuclear inflammatory cells adhered to the valve (arrows) (X100); (**d**) spleen with multiple melanin deposits (arrowheads) and melano-macrophage centres (arrows), e = ellipsoids (X200); (**e**) spleen, congested with multiple confluent, circular granuloma-like inflammatory lesions (*) (X40); skeletal muscle showing skeletal muscle fasciitis (*) and myositis and perivascular inflammation in the dermis (*). Scalebar = 500 microns.

Sentinel control fish (in a separate tank, within the same flow-through system) displayed minor pathology indicating fish were responding to the presence of a pathogen, but no Gram-Glynn-positive cocci were detected in any organs or tissues. PCR analyses supported these results, since all sentinel fish in replicate 1 tested PCR-negative for *S. agalactiae*. All four fish had mild cholangiohepatitis, with some perivascular inflammation (Fig. 29a). A few fish had mild pericarditis (Fig. 29c), and the bulbus arteriosus of one fish had infiltrates of monoculear inflammatory cells into the myoelastic and connective tissues (Fig. 29b). One fish had a mild branchitiis, two fish had a mild interstitial nephritis, one fish had very mild choroiditis. One fish had a mild pancreatitis and steatitis, with perivascular inflammation of the mesenteric vessels (Fig. 29d). The spleen, head and caudal kidney of three fish had an increased number of melano-macrophage centres.

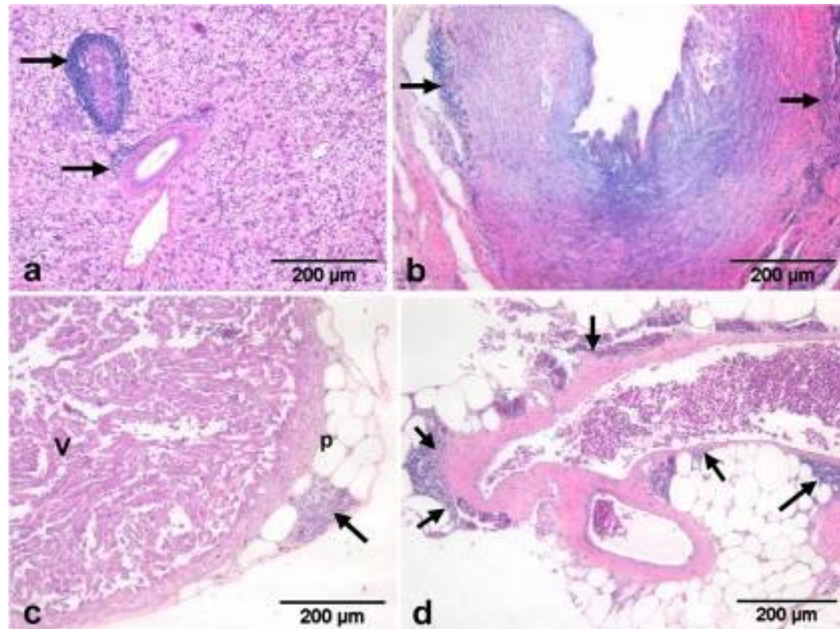


**Figure 29.** Micrographs of histological sections of the liver, heart and mesenteric vessels from Qld grouper from the immersion challenge trial (sentinel control, replicate 1) stained with H&E**;** (**a**) Liver showing inflammation of bile ducts (arrows) (X100); (**b**) bulbus arteriosus with mononuclear inflammatory infiltrate into the myoelastic and connective tissues (arrows) (X100); (**c**) Ventricle (v) of the heart with focal inflammation of pericardium (p) (arrow) (X100); (**d**) mesenteric vessel in peritoneum with perivascular inflammation and steatitis (arrows) (X100).

Positive control fish (four fish injected with $10^6$ cfu *S. agalactiae* fish$^{-1}$) had pathology indicating bacterial septicaemia from streptococcosis, with widespread organ involvement. All four fish had a marked granulomatous inflammatory response, with aggregations of macrophages containing Gram-Glynn-positive cocci in multiple organs and tissues. Pathology was similar to that already described for the injection challenge trial. Negative control fish (four fish injected with sterile PBS, and maintained in a separate holding system) were all negative for *S. agalactiae*. No Gram-Glynn positive cocci were visible from examination of Gram-Glynn stained tissue sections.

***Replicate 2***
In the low dose treatment group, all four fish histologically examined, displayed a mild, mixed inflammatory response, in the eye, heart, spleen, liver, gills, peritoneum and skin. All fish had mild iritis, choroiditis, pericarditis, splenic congestion, serositis, branchiitis, dermatitis and hepatitis. Gram-Glynn-positive cocci were occasionally detected in macrophages; in the eye choroid; sclera connective tissues; in sloughed macrophages between adjacent gill lamellae, or near the epidermal surface of the skin or fins. One fish had a marked hepatitis.

In the medium dose treatment group, two of four fish histologically examined, had evidence of a systemic bacterial infection, with multi-organ involvement. Gram-Glynn-positive cocci were detected in the eyes, heart, brain, spleen, gills, peritoneal cavity, stomach and fascia of skeletal muscle (Fig. 30a, b, c, d). Two fish had a moderate to severe dermatitis of the fins with hyperaemia. The head kidney, and spleen had an increased number of melano-macrophages.

In the high dose treatment group, all four fish histologically examined, had evidence of a systemic bacterial infection, with multi-organ involvement. Gram-Glynn-positive cocci were

detected in the brain, eyes, skin, fins, heart, kidney, liver, spleen, intestine, stomach, pancreas, peritoneal adipose tissue and skeletal muscle. All four fish had ophthalmitis, with marked choroiditis, iritis, keratitis and cellulitis of extra-ocular connective tissues, the choroid most affected. All four fish had meningitis, with Gram-positive cocci visualised on Gram-Glynn special stain.

Fish with red, hyperaemic skin lesions had myositis, dermatitis and cellulitis. Large numbers of Gram-Glynn-positive cocci were detected among degenerating muscle fibres and fascia. All fish had marked dermatitis of the fins, with oedema and haemorrhage (as described earlier). All four fish had branchiitis, and a moderate to severe pericarditis, with inflammatory lesions extending deeper into the ventricular myocardium of the heart. All four fish had splenitis, with loss of ellipsoid structure, replaced with fibrous granulomatous inflammatory cells and fibrous tissue. All fish also had mild cholangiohepatitis.

The stomach of all four fish had diffuse necrosis of the gastric mucosal epithelium, with Gram-positive cocci in goblet cells of gastric glands and in sloughed epithelium. There was scattered focal necrosis in the lamina propria and submucosal layers, and marked granulomatous inflammation with Gram-Glynn-positive cocci in loose connective tissue layer of muscularis layer. The intestines had foci of haemorrhagic enteritis and colonies of bacteria in the mucosal epithelium. The lamina propria of the pyloric caecae was largely dilated, with oedema and colonies of Gram-Glynn-positive cocci & dilated blood vessels. There was enteritis, with sloughing of mucosal epithelium and macrophages into the intestinal lumen, and colonies of Gram-Glynn-positive cocci in the lumen. The serosa was often thickened with a diphtheritic-like membrane made up of granulomatous inflammatory cells.

All four fish histologically examined from the sentinel treatment group (in a separate tank, within the same flow-through recirculation system), had pathology indicating fish were responding to presence of a bacterial pathogen. All fish had a mild branchiits, pericarditis, choroiditis, iritis, or dermatitis of the fins. Two fish had pathology evidence of infection with *S. agalactiae*, with Gram-Glynn-positive cocci detected inside tissue macrophages; in the meninges and third ventricle of the brain of one fish, and in the peritoneal cavity in the other fish. The spleen, head and caudal kidneys of all four fish had increased number of melano-macrophages.

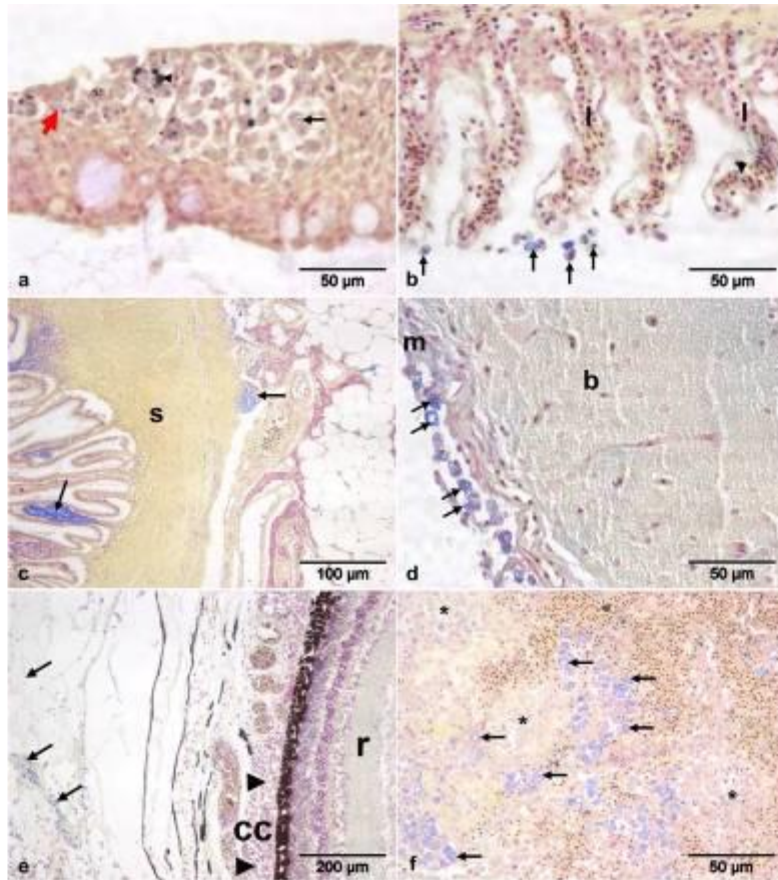


**Figure 30.** Micrographs of histological sections of the skin, gills, intestine, brain, eye and spleen from juvenile Qld grouper from the Immersion challenge trial (medium dose, replicate 2) stained with Gram Glynn; (**a**) skin with necrotic epithelial cells (red arrow, arrowhead) and a macrophage with *S. agalactiae* (arrow) (X400); (**b**) gill lamellae showing multiple sloughed macrophages containing blue-staining *S. agalactiae* (arrows) (X400); (**c**) intestine with bacteria in lamina propria (long arrow) and in the serosa (short arrow) (X200); (**d**) brain (b) with multiple Gram-Glynn-positive cocci in macrophages in the meninges (m, arrows) (X400); (**e**) choroid; note bacteria (arrows) in choroid space, cc = choroid capillaris, r = retina (X100); (f) spleen showing multiple macrophages containing Gram-Glynn-positive cocci (arrows) among ellipsoids (asterisks) (X400).

## Conclusions and Discussion

The combined laboratory results from experiment 2 (injection challenge trial, replicates 1 & 2), fulfilled Koch's postulates. The experiment successfully demonstrated that the *S. agalactiae* bacterial strain QMA0825, originally isolated from the kidney of a wild Qld grouper that died in Townsville in 2010, is highly virulent to juvenile grouper, *E. lanceolatus*. *S. agalactiae* bacterial strain QMA0825, when injected into fish, at various dose rates, resulted in overt clinical signs of streptococcosis and death within three to four days, causing nearly 100% mortalities, with a marked dose effect at higher doses. The combined laboratory test results, including bacteriology, PCR and histopathology, from the injection trial (both replicates), confirmed that 98% of fish injected with *S. agalactiae* became infected, developed clinical signs of streptococcosis and died within three to four days. Juvenile grouper showed overt clinical signs and pathology typical of infection with *S. agalactiae*, as reported in the literature (Chen *et al*. 2007; Filho *et al*. 2009; Abuseliana *et al.* 2011). There was also some pathology that has not been previously been described for fish infected with *S. agalactiae,* and some other, more unusual pathology (not described here).

The combined laboratory test results from experiment 3 (the immersion challenge trial, replicates 1 & 2), demonstrated that *S. agalactiae* can spread through the water and infect juvenile grouper, regardless of the dose of bacteria present in the water. Pathology results showed 50% of fish (from the three treatment groups) in replicate 1, and 75% fish from replicate 2, were positive for *S. agalactiae*. In contrast to experiment 2, (where fish were injected with the bacteria), juvenile grouper from experiment 3, showed a milder inflammatory response, and developed infection at a much slower rate. Furthermore, all surviving fish (except 1), showed no overt clinical signs of infection, but many fish were positive by PCR, bacteriology or pathology. Special histological stains enabled visualisation of Gram-positive cocci in multiple organs and tissues, at the cellular level, inside tissue macrophages, adhered to erythrocytes and adhered to the endothelial lining of blood vessels, thus confirming infection. The bacteria were also detected in replete macrophages that had been shed by the fish, in macrophages on the epithelial surface of the skin, in the gills or on the intestinal mucosal surface. This indicated fish had mounted an innate immune response to the bacterium, and were sub-clinical carrier fish.

Detailed histological analysis of fish from experiments 2 & 3 (the injection and immersion challenge trials), enabled detection of *S. agalactiae* bacteria at the cellular level. Histopathology observations made during this study, show macrophages played a role in the immune response of juvenile Queensland grouper to Gram positive bacteria such as *S. agalactiae*. *S. agalactiae* bacteria were often detected in intact (not necrotic) macrophages, often within the cell cytoplasm, in the phagosomes of macrophages in; the meninges and third ventricle of the brain, the pericardium and endothelial cells of the heart; the epidermis and dermis of the skin; the eye choroid; spleen; head and caudal kidney haematopoietic tissue; liver, connective tissues of the swim bladder and eye; in macrophages in the gill lamellae; in the mucosal epithelium of the intestine; in peritoneal macrophages, in both moribund and in surviving fish (from both the injection and immersion challenge trial).

Fish macrophages and leukocytes are known to play a crucial role in the innate immune response of fish, as a first line of defence against invading pathogens, with macrophages phagocytosing and destroying bacterial pathogens. Histopathology observations suggest resident macrophages exist in the gills, intestine and skin of grouper, and may play an active role in the innate immune response of Qld grouper against invading *S. agalactiae*. It is possible *S. agalactiae* has certain mechanisms enabling it to remain intact, (once engulfed) inside macrophages, intracellularly within phagosomes (as was commonly observed from detailed histopathology observation), and may avoid apoptosis. In this way, *S. agalactiae* may be able to avoid the host's further humoral immune response, and be transported around the body, to various organs and tissues, and to cross the blood brain barrier, gaining entry into the brain. This is similar to what has been earlier described for *S. iniae* in mammals, ie. the '*Trojan horse effect'* (Zlotkin *et al*. 2003). A more recent study, using gene expression to investigate the virulence of a piscine *S.agalactiae* in host-pathogen interactions, used a mouse model, with *in vitro* cell infection, and evaluated various pathogenic characterisitcs of a beta-haemolytic strain of *S.agalactiae* isolated from diseased tilapia in China (Goa *et al*. 2014). This study indicated that internalisation of *S.agalactiae* by murine macrophages, resulted in major changes in the gene expression, associated with signal transduction mechanisms and transcription, activating various mechanisms, allowing for survival of *S.agalactiae* intracellularly. Further immunological, and molecular genetic research, using *in vitro* fish cell, would be useful to investigate various pathogenic mechanisms utilised by *S.agalactiae* in Qld grouper, for intracellular survival in macrophages and host survival.

## 6.7 Development of oral and cohabitation challenge models in Queensland grouper *E. lanceolatus* and histopathology analysis

### 6.7.1 Methods

**Experimental design and fish husbandry**

Juvenile Queensland grouper (*E. lanceolatus*), weighing approximately 30 g, were obtained from the Northern Fisheries Centre in Cairns (Animal Science, Agri-Science Queensland, DAFF) and held in full strength sea water at 35 ppt salinity in a 100 L fibre glass aquaria. Fish were allowed to acclimate for four weeks in two separate recirculating quarantine aquarium systems, prior to the experiments.

Aquaria were organized into two systems (replicates 1 and 2), each consisting of eight tanks (160 L) each. Each replicate system was located in a separate room (Figure 1). Each replicate system was supplied by a separate recirculation system, consisting of 1 x 200 L sump, 50 L bio-filter, and a protein skimmer. The temperature of both systems was maintained at 28°C by three heating and cooling systems, with a recirculation rate of approximately 120 L per hour. A 15% water exchange was performed every week and water quality was checked on a weekly basis for ammonium, nitrite and pH level, for each replicate system.

**Experiment 4: oral challenge trial**

Two tanks per replicate system, were assigned for each treatment group (high or low dose). Ten fish were randomly assigned to each treatment group (Fig.1). Fish were fed pelleted food, mixed with either a high dose of bacteria ($10^6$ cfu bacteria fish$^{-1}$), or a low dose of bacteria ($10^3$ cfu bacteria fish$^{-1}$). Pelleted feed containing *S. agalactiae* (QMA0285) was prepared daily as follows. Commercial feed (Ridley Aqua feed: 4mm marine floating pellets) was autoclaved to ensure no other bacteria were present. Every day a fresh overnight THB culture of QMA0285 was obtained in the stationary phase, and standardised to an optical density at 600 nm of 1.0 (OD600) (equivalent to 1-3 x $10^8$ cfu mL$^{-1}$). Then, using a high quality fish oil provided by Ridley Aqua feed, emulsions of the bacterial suspension were prepared at two different concentrations; $10^8$ cfu mL$^{-1}$ and $10^5$ cfu mL$^{-1}$, and coated onto the feed (approximately 25 pellets/fish/day) by vortexing the mixture until all the emulsion was absorbed. The resulting coated feed was determined by viable count to contain approximately $10^6$ cfu fish$^{-1}$, or $10^3$ cfu fish$^{-1}$ for the two respective treatments. Two replicate cohorts of ten fish per treatment were used.

A control group of ten fish were assigned to each replicate (for both experiments). These control fish were fed on a maintenance diet comprising a commercial 4mm floating diet (Ridley Aqua Feed, Narangba, Queensland), equivalent to 5% body weight, for a period of five weeks, until the experiment was terminated.

**Experiment 5: co-habitation challenge trial**

Three tanks per replicate system were assigned for each treatment group. Fish were randomly assigned to each treatment group, according to the treatment group. ie. ratio of infected (cohabitant) fish to uninfected (cohabitee fish) (Fig. 31).

All juvenile grouper were fed once a day, with a maintenance diet comprising a commercial 4 mm floating diet (Ridley Aqua Feed, Narangba, Queensland), equivalent to 5% body weight, for a period of five weeks, until the experiment was terminated.

Infected (cohabitant) fish were challenged by intra-peritoneal injection (100 µL to $10^6$ cfu

fish$^{-1}$), dose determined from the injection challenge. Infected cohabitant fish were introduced into duplicate tanks containing uninfected (healthy) cohabitee fish.

Three ratios of cohabitants to cohabitees were tested; 1:5, 1:2 and 1:1 (in duplicate). In all cases fish were given 24 h care during the challenge period. Any fish showing gross clinical signs of disease (exophthalmia, erratic swimming or darkening of the skin) was immediately euthanased by overdose of fish anaesthetic (AQUI-S) in accordance with animal welfare and ethics requirements, and counted as a mortality. Mortalities were recorded every 4 h for the first seven days of the experiment. As mortality rates were very low or nil, experiments were maintained for five weeks and fish were checked twice daily for any clinical signs of ill health. At the end of the challenge trial, all remaining fish were euthanased by immersing fish in AQUI-S at a dose rate of 40 mg L$^{-1}$.

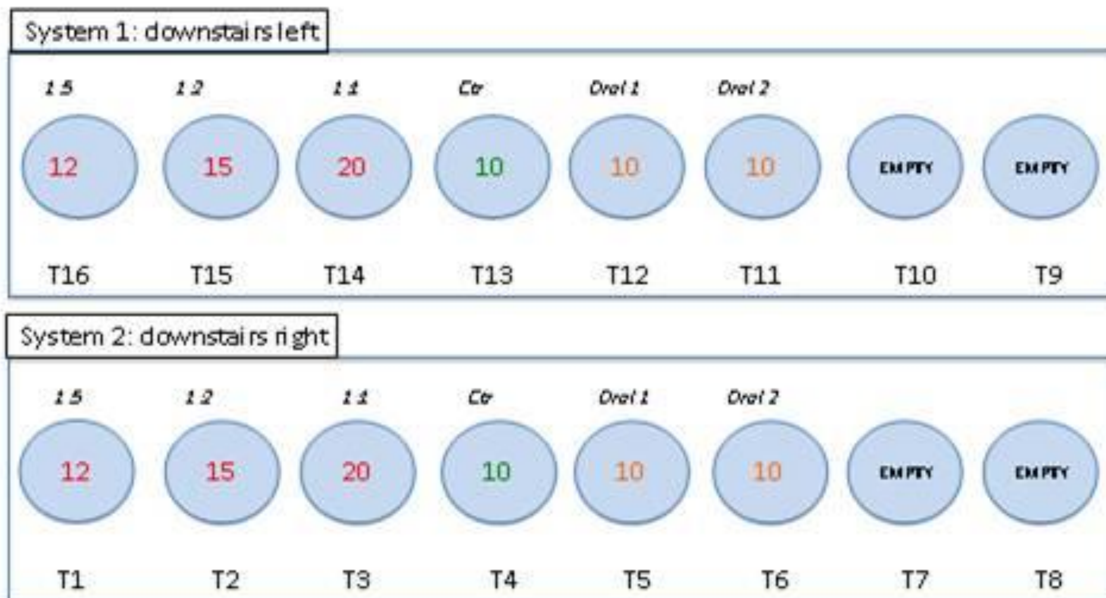


**Figure 31.** Experimental design for the Oral and Cohabitation Challenge Trials. System 1 = Replicate System 1. System 2 = Replicate System 2. T = tank number, Ctr = Control, Oral 1 = high dose ($10^6$ cfu bacteria fish$^{-1}$), Oral 2 = low dose ($10^3$ cfu fish$^{-1}$)

**_S. agalactiae_ strain and growth conditions**

Similar to the injection & immersion challenge trials, *S. agalactiae* (QMA0285) isolated from the head-kidney of a wild Queensland grouper, Cairns (2010), was used for both oral and cohabitation challenge experiments. The isolate was retrieved from stock (-80°C) and cultured on sheep blood agar (SBA) (Oxoid) for 48 hrs at 28°C. From pure culture, a single colony forming unit (cfu) was inoculated to 5 mL Todd Hewitt Broth (THB) and incubated overnight at 28°C with 100 rpm agitation. Identity following broth culture was confirmed by direct lysis, PCR amplification using the AgaF/AdyR primer pair as described previously.

**Preparation of bacterial inocula**

The bacterium was grown overnight growth in THB at 28°C with continual shaking to provide bacteria in the exponential growth phase. The bacterium was then identified by PCR

analysis, from a subsample 100 µL aliquot. The remaining 4.9 mL was pelleted by centrifugation 5 min at (4000 x *g*) and washed twice in PBS. The pellet was then resuspended in an appropriate volume of PBS until a final optical density of 1.0 was reached at $A_{600}$ (absorbance at 600 nm wavelength). An OD = 1 for GBS is estimated to be between 1 to 3 x $10^8$ cfu $mL^{-1}$. This standard cell suspension was diluted 10-fold in sterile PBS to obtain $10^7$ cfu $mL^{-1}$. The final concentration was determined post-hoc by viable count on SBA.

**Bacteriological analysis and confirmation by PCR analysis of QMA0285 from bacterial cultures**

For the oral challenge trial (both replicates), six fish from each treatment group were analysed bacteriologically for *S. agalactiae*. Bacterial isolation was done on dead or euthanased fish on samples of internal organs; including the brain, kidney, liver and spleen. Organs were dissected using aseptic techniques, streaked onto SBA, and incubated at 28 °C for up to 3 days. From pure culture on SBA, QMA0285 showed typical phenotype of GBS (non-haemolytic, opaque, mucoid in appearance and adhered strongly to the blood agar). Samples from positive growths were collected using a 1 µL sterile loop and resuspended in PCR tubes (200 µL, SSI) containing 50 µL of ultra pure DNAse, RNAse free water (Gibco). DNA templates were prepared from both bacterial cultures and organs and were subjected to PCR analysis as described previously. When no growth occurred, regions of the SBA plate were still swabbed for PCR.

Bacteria with phenotypes differing from typical GBS grew well on SBA. Samples of representatives of each phenotype were collected, and their DNA extracted. To identify the bacteria to species level, the 16S rRNA region was amplified and sequenced using the 27F and 1492R primers by standard techniques.

**PCR analysis on whole tissues**

Fish from both oral and cohabitation challenge trials were sampled for detection of *S. agalactiae* by PCR. Internal organs from each fish, including the brain, head kidney, and spleen were sampled from each fish, from each treatment group (both replicates). Samples were placed into absolute ethanol and kept at 4°C until processing. PCR analysis was done on individual organs as described previously. Tests were undertaken on individual and pooled organs from each fish. Tests on pooled organs were used to give an overall test result for each fish. A minimum of one positive organ test result was required for a fish to be considered positive for *S. agalactiae* by PCR. Test results (for individual organs and pooled organs), for both the oral and cohabitation challenge trials, including control treatment groups are shown in Appendices 7, 8 & 9.

**Histopathology**

Fish from all treatment groups, from both the oral and cohabitation challenge trials, were sampled for histology. Organs including the brain, gills, eye, heart, head kidney, caudal kidney, spleen, liver, muscle, skin, stomach, intestine, pyloric caecae and swim bladder were sampled from all dead and moribund euthanased fish. In the oral challenge trial (both replicates), six fish from each treatment group were analysed. Organs were fixed in 10% formalin for 48 h, and transferred to 70% ethanol. All samples were stored at 4°C until processed for histology. Sections (5 µm) were stained with haematoxylin and eosin (H&E). Special Stains including Gram Glynn and Brown and Brenn Gram stain and Ziehl-Neelson, were prepared according to Bancroft and Stevens (1992).

Detailed histopathology examination of H&E, Gram Glynn (and all special stains) stained

slides of each organ and tissue, were examined in detail, for a minimum of 20 min per slide, using an Olympus BX41 compound light microscope. A fish was considered positive for *S. agalactiae* by histopathology analysis, if Gram-positive-cocci were visualised (analysing Gram-Glynn special stains) inside any organ or tissue, and if the fish also showed pathology indicative of infection. Pathology indicative of infection ranged from mild, to moderate to severe changes, including inflammation of the eye, heart, brain, liver, head or caudal kidney, spleen, gills or other organ or tissue. Representative lesions indicative of streptococcosis, were photographed from both challenge trials (as described in section 6.6).

**Statistical analysis for comparison of PCR, histology and bacteriology tests**
The McNemar test was used for statistical analysis, to compare test results using the three different test methods (PCR, histology and bacteriology) employed to detect *S. agalactiae* in fish from both experiments. This was done for fish from both the oral and cohabitation challenge trials, for each replicate system, since there was not always agreement with test results. The McNemar test relies on comparing test sample results that do not have agreement. This was done to compare the sensitivity of each test. ie. which test picks up more positives from the same fish tested by the three different test methods. Pooled samples for each fish were used, ie. a fish was considered positive, if the brain, heart or spleen tested positive by PCR, bacteriology or Gram Glynn special stain in histology analysis (Appendices 7, 8 & 9).

### 6.7.2 Results and Discussion

**Confirmation of GBS identity by PCR**
Strain QMA0285 was confirmed to be Group-B *Streptococcus* by PCR using primer pair AgaF/AdyR (Fig. 32) before being used to inject fish in the cohabitation challenge and was checked routinely for the oral challenge before being coated onto the feed.

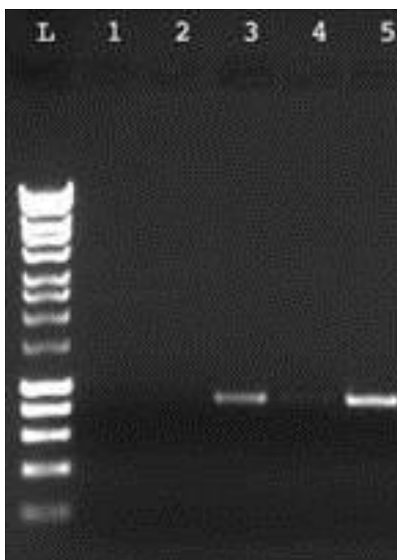


**Figure 32.** GBS identify confirmation by PCR using AgaF/AdyR primer pairs. AgaF and AdyR are specific for *S. agalactiae*. For each reaction 3 µL of PCR product were mixed with 2 µL of loading buffer and loaded onto a 1% agarose gel; L: 5 µL of markers (HyperLadder, Bioline). 1: negative control using genomic DNA from *S. iniae*; 2: no DNA, only ultra pure water; 3: DNA from strain QMA0285 isolated from a wild Queensland grouper in Cairns, 2010 (neat); 4: DNA from strain QMA0285 (1/10 dilution); 5: positive control using known GBS's high molecular genomic DNA from strain QMA0326 (1/100 dilution) isolated from Estuary ray *Dasyatis fluviorum* (*Sea World*).

## Experiment 4: oral challenge trial

### *Clinical signs*

Oral challenge resulted in development of clinical signs of disease in approximately 10% of fish challenged with the highest dose ($10^6$ cfu fish$^{-1}$) of *S. agalactiae*. There was a delayed onset of development of clinical signs. Two fish, from the high dose treatment group, displayed clinical signs of infection on day 26. In replicate 1, a single fish (fish 1) was moribund and observed immobile at the bottom of the tank, with severe unilateral exophthalmia of the right eye, and ascites. In replicate 2, a single fish (fish 1) was swimming in a disoriented manner, with darkening of the skin and loss of appetite. This fish became anorexic, and remained at the bottom of the tank, until the end of the experiment. External clinical signs of infection were rarely observed in other fish in the high dose treatment groups (in both replicates), and included reddening of the fins and a small or reduced size spleen or liver (Appendix 7).

In contrast, fish fed on the low dose of *S. agalactiae,* showed few or no clinical signs of infection until day 33. In replicate 1, two fish showed anorexia and red discoloration of the caudal fins, whereas in replicate 2, all fish had a full stomach, with no visible external signs of infection until the end of the experiment. All other fish survived the five week duration of the experiment until euthanasia at the end of the experiment.

### *Bacteriology*

*S. agalactiae* was detected by bacteriology in two of six fish (33%) from the high dose treatment groups (replicates 1 & 2), and in one fish (17%) (replicate 1) from the low dose treatment group. *S. agalactiae* was not detected from any fish from the control groups, both replicates 1 and 2 (Appendix 7).

Isolation of bacteria other than GBS

Phenotypes different from GBS were observed growing on SBA from samples collected during both the oral and cohabitation challenge trials. These phenotypes were described according to their shape, surface/edge, colour, haemolysis and size characteristics. Representatives of each phenotype were chosen and putative identities described by partial sequencing of the 16S rRNA gene (Table 25).

**Table 25.** Putative identities of bacteria that were observed growing on SBA revealed by partial 16S rRNA gene sequence.

| Phenotype | Genus | specie | Sequence length (bp) | Max identity |
|---|---|---|---|---|
| A | *Streptococcus* | *agalactiae* | 1367 | 100% |
| B | *Photobacterium* | *damselae* | 1259 | 99% |
| C | *Vibrio* | *owensii* | 1359 | 99% |
| D | *Shingobium* | *abikonense* | 1285 | 100% |
| E | *Vibrio* | *alfacsensis* | 1102 | 100% |
| F | *Shewanella* | *putrefaciens* | 1346 | 99% |
| G | *Pseudomonas* | *pachastrellae* | 1387 | 99% |
| H | *Enterococcus* | *gallinarum* | 1368 | 100% |

Note: all bacteria listed are Gram-negative, rod-shaped bacterium except *E. gallinarum,* which is a Gram-positive streptococcus type D; base pair: bp.

These bacteria are known marine bacteria that are naturally present in the water column. It is not uncommon to find bacteria such as *Vibrio* sp, *Photobacterium* sp. *Pseudomonas* spp as part of the normal flora of the gills, mucosal or gut bacterial community. However, these bacteria were abnormally isolated by bacteriology from the liver, spleen, head-kidney or brain of several fish in the oral and cohabitation trials, suggesting that the immune system of these fish was compromised. The origin of these bacteria is unclear but most likely they would have been brought into the system with the fish. Bacteria can be carried sub-clinically as part of the fish commensal bacterial community. In a stressful situation such as the introduction of a pathogen, (such as in these experimental challenge trials) or during acute changes in environmental conditions, the fishes' immune system will be challenged and can lead to disequilibrium of the normal immune homeostasis between commensal and pathogenic microbes. Fish can harbour these pathogens as subclinical infection and become carriers for long time periods.

### PCR

Amplicons of *S. agalactiae* were detected by PCR in 96% fish, from both the high and low dose treatment groups, (replicates 1 & 2) (Appendix 7). Three fish from the control groups tested positive for *S. agalactiae*; two fish from replicate 1, and one fish from replicate 2. Individual test results for each organ tested from each fish, and pooled results (for each fish) are shown in Appendix 7.

### Histopathology summary

*S. agalactiae* was detected by histopathology examination from ten fish out of a total of 36 fish examined (Appendix 7). Gram-Glynn-positive cocci were detected, variously in organs from each of these fish; in the brain, spleen, heart, gills, kidney or spleen. The following fish tested positive for *S. agalactiae* by histopathology; one fish (17%) from the high dose treatment group (replicate 1); 3 fish (50%) fish from high dose treatment group (replicate 2); five fish (87%) from low dose treatment group (replicate 1); and one fish (17%) from the control group (replicate 2) (Appendix 7).

### Laboratory Test Results - Summary

A summary of all laboratory test results for individual fish indicated that juvenile grouper can become infected with *S. agalactiae* by ingestion of *S. agalactiae*-contaminated food. Fish fed either low ($10^3$ cfu/fish) or high ($10^6$ cfu/fish) doses of bacteria, tested positive for *S. agalactiae,* by PCR, bacteriology or histology (Table 26). Test results for individual fish and organs are shown in Appendix 7.

**Table 26.** Summary of all laboratory test results (PCR, Bacteriology, and Histology) used to detect *S. agalactiae* in juvenile Queensland grouper, *E. lanceolatus*, from the oral challenge trial. R1 = Replicate 1; R2 = Replicate 2; High dose = fish fed with $10^6$ cfu fish$^{-1}$; Low dose = fish fed with dose $10^3$ cfu fish$^{-1}$. PCR was done on whole organs at TAAHL, histology at TAAHL, bacteriology done at UQ.

| Replicate: | R1 | R1 | R1 | R2 | R2 | R2 |
|---|---|---|---|---|---|---|
| Test (Laboratory) | High Dose (n=6) | Low Dose (n=6) | Control (n=6) | High Dose (n=6) | Low Dose (n=6) | Control (n=6) |
| PCR (TAAHL) | 6 | 6 | 2 | 5 | 6 | 1 |
| Bacteriology (UQ) | 2 | 1 | 0 | 2 | 0 | 0 |
| Histology (TAAHL) | 1 | 5 | 0 | 3 | 0 | 1 |

**Histopathology interpretation of juvenile Queensland grouper examined from the experiment 4, the oral challenge trial (replicate 1)**

***Control fish***

Histopathological examination of organs and tissues from six fish from the control group, replicate 1, showed all were negative for *S. agalactiae*. No bacteria were detected from detailed histological examination of all H&E and all Gram-Glynn stained organs and tissues, from all fish from the control group (Appendix 7). Two fish had a bacterial infection with a Gram-negative, rod-shaped bacterium. The bacterium causing the lesions may have been one of a number of Gram-negative bacteria identified by PCR (see Table 25). These fish displayed mild branchitis, interstitial nephritis, mild pericarditis, myocarditis and mild hepatitis. Special histological stains including Ziehl-Neelson, Brown & Brenn Gram stain and Gram Glynn demonstrated Gram-negative rod bacteria in the centre of multiple granulomas, in the head and caudal kidney of these fish with a mild interstitial nephritis (Fig.33b, c & d). The caudal kidney contained a few large melano-macrophage centres (Fig 33a).

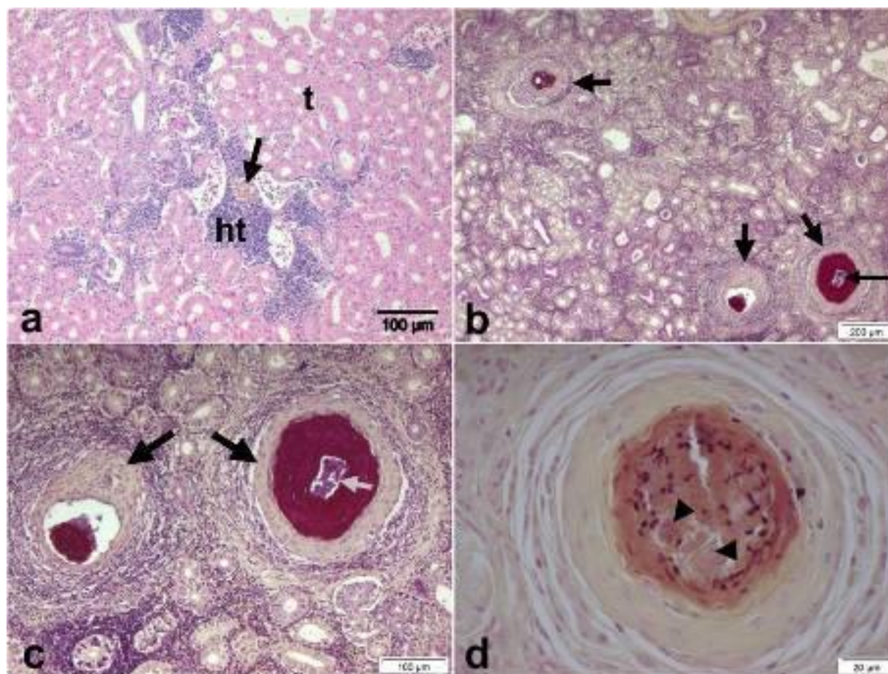


**Figure 33.** Micrographs of histological sections of caudal kidney and liver from juvenile grouper *E. lanceolatus*, from the oral challenge trial (control group, replicate 1). (**a**) caudal kidney with melano-macrophage centres (arrow) among the haematopoietic tissue (ht), t=kidney tubules (X200) H&E; (**b**) caudal kidney containing numerous bacterial granulomas (arrows) Long arrow (lower right hand side), points to central core of granuloma, containing Gram-negative bacteria (X100) Brown & Brenn; (**c**) higher power view of (b) showing Gram-negative, rod-shaped bacteria in the central core of the granulomas (X200) Brown & Brenn; (**d**) Colonies of Gram-negative, rod-shaped bacteria detected in the central core (arrowheads) of the granulomas (X1000) Brown & Brenn.

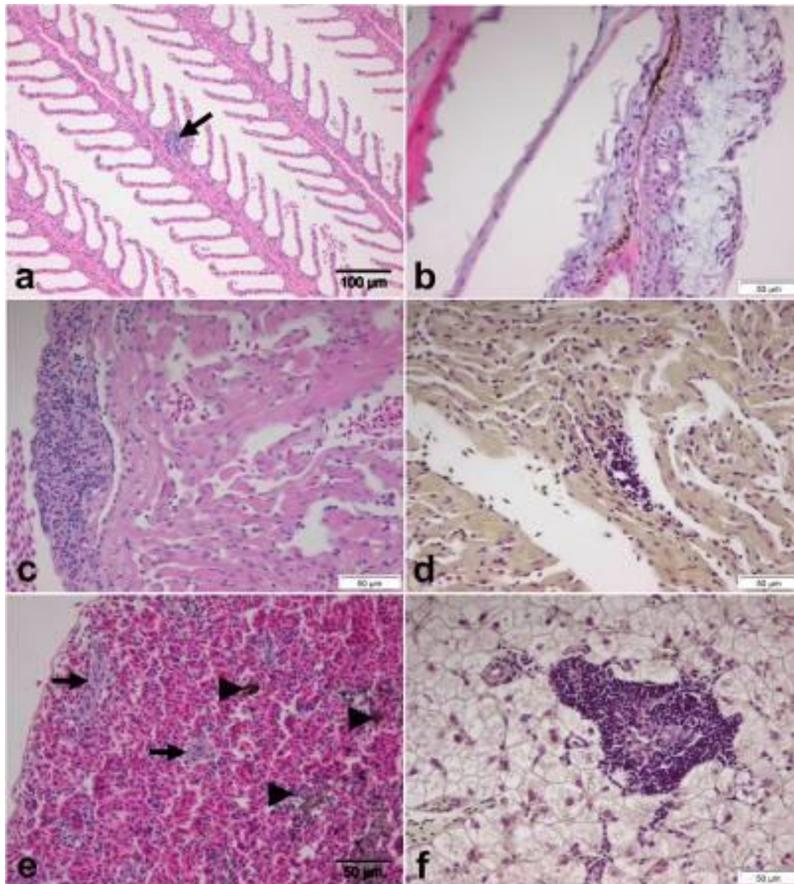


**Figure 34.** Micrographs of histological sections of the gills, skin, heart, spleen and liver from juvenile grouper *E. lanceolatus*, from the oral challenge trial (control group). (**a**) gills with rare foci of inflammatory cells between adjacent gill lamellae (arrow) (X200) H&E; (**b**) skin with epidermal spongiosis (X400) H&E; (**c**) heart ventricle with focal inflammation of pericardium (X200) H&E; (**d**) cardiac muscle with focal myocarditis (X400) Brown & Brenn; (**e**) spleen with multiple foci of melanin (arrowheads) and normal ellipsoids (arrows) (X400) H&E; (**f**) liver with focal inflammatory lesion (X400) Brown & Brenn.

The gills of all six fish had a very mild branchitiis. Gill lesions were rare, and consisted of aggregations of mononuclear inflammatory cells at the base of the gill filaments (Fig. 34a). Gill lamellae were occasionally thickened, with epithelial hyperplasia of a few adjacent gill filaments. The heart of two fish had mild pericarditis with multifocal lesions consisting of aggregations of mononuclear lymphocytic inflammatory cells in the pericardium of the ventricle (Fig. 34c), atrium or bulbus arteriosus. A few fish had mononuclear inflammatory cells infiltrating into the myocardium of the ventricle or atrial cardiac muscle (Fig. 34d), or into the myoelastic tissue of the bulbus arteriosus. All other fish had normal hearts with no lesions. The spleens of all fish had melanin scattered throughout the red and white pulp (Fig. 34e). The livers of most fish were normal, with good lipid storage and well-vacuolated hepatocytes. One fish had a mild hepatitis, with multiple focal aggregations of mononuclear inflammatory cells (Fig. 34f).

**Low Dose**

Examination of six fish from the low concentration group (replicate 1) showed five of six fish (fish 1, 3, 4, 5 and 6) were infected with *S. agalactiae* (Appendix 7). The remaining fish was a suspect positive, with Gram-Glynn-positive cocci visualised in sloughed macrophages between individual gill filaments. One fish had a systemic bacterial infection with multiple organ involvement including the head and caudal kidney, spleen, and swim bladder. Gram-

Glynn-positive cocci were visualised in a granuloma in the caudal kidney. All six fish had a very mild branchitiis (as described for control fish). Four fish had a very mild pericarditis, with multifoci of mononuclear inflammatory cells in the pericardium of the ventricle. The spleens and livers of all six fish had changes similar to that already described for the control fish. Many fish had numerous, large, melano-macrophage centres in the head and caudal kidney.

**High Dose**

One fish was infected with *S. agalactiae* with marked pathology. Gram-Glynn-positive cocci were visualised in all organs and tissues, including the gills, brain, eyes, head and caudal kidney, spleen, heart and liver. Gram-Glynn-positive cocci were detected in the choroid of the eye, in the peri-scleral connective tissues, and adhered to the scleral cartilage (Fig. 35a, b). Gram-Glynn-positive cocci were visible in the meninges of the brain, and in a large, granulomatous inflammatory lesion in the third ventricle (Fig. 35c, d, e & f).

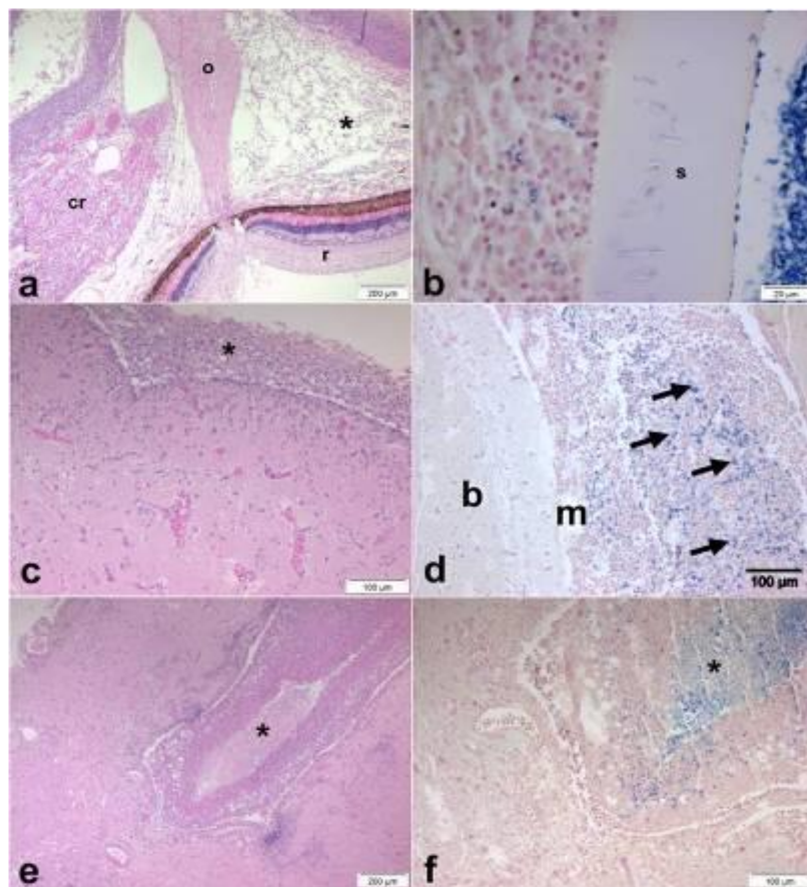


**Figure 35**. Micrographs of histological sections of the eye and brain from a juvenile grouper *E. lanceolatus*, from the oral challenge trial (high dose treatment group). (**a**) eye showing inflammation of the choroid rete (cr) and choroid space (*) r = retina, o = optic nerve (X100) H&E; (**b**) eye sclera. Note the Gram-Glynn-positive cocci (blue staining) visible among inflammatory infiltrate in the episcleral connective tissue, and adhered to scleral cartilage (s) (X1000) Gram Glynn; (**c**) brain with meningitis. Note thick layer of mixed inflammatory infiltrate (*) in meninges (X200) H&E; (**d**) brain meninges (m) showing mixed inflammatory infiltrate with macrophages containing Gram-Glynn-positive (blue) cocci (arrows), b=brain, m=meninges (X200) Gram Glynn; (**e**) Third ventricle of the brain is filled with a thick layer of granulomatous inflammatory cells (*) (X100) H&E; (**f**) Higher power view of (e) showing the mixed inflammatory infiltrate and macrophages (*) containing Gram-Glynn-positive cocci (X200) Gram Glynn.

One fish (fish 4) had a bacterial infection with Gram-negative bacteria, with multiple bacterial granulomas in the kidney. The other five fish had mild branchiitis, choroiditis, iritis, pericarditis, and hepatitis, the spleen, liver and kidney had changes (as previously described for the low dose fish), but no bacteria were visualised from examination of Gram Glynn special stains.

**Control fish (replicate 2)**
Histopathology examination of six fish from the control group showed one fish was infected with *S. agalactiae*, with a single macrophage containing Gram-positive bacteria detected in the ventricular lumen. This fish also had pericarditis, focal myonecrosis of the bulbus arteriosus and atrium, and endocarditis (Fig. 36a, b, c, d). The swimbladder was inflamed (Fig. 36f), and there was a mild hepatitis (Fig. 36e).

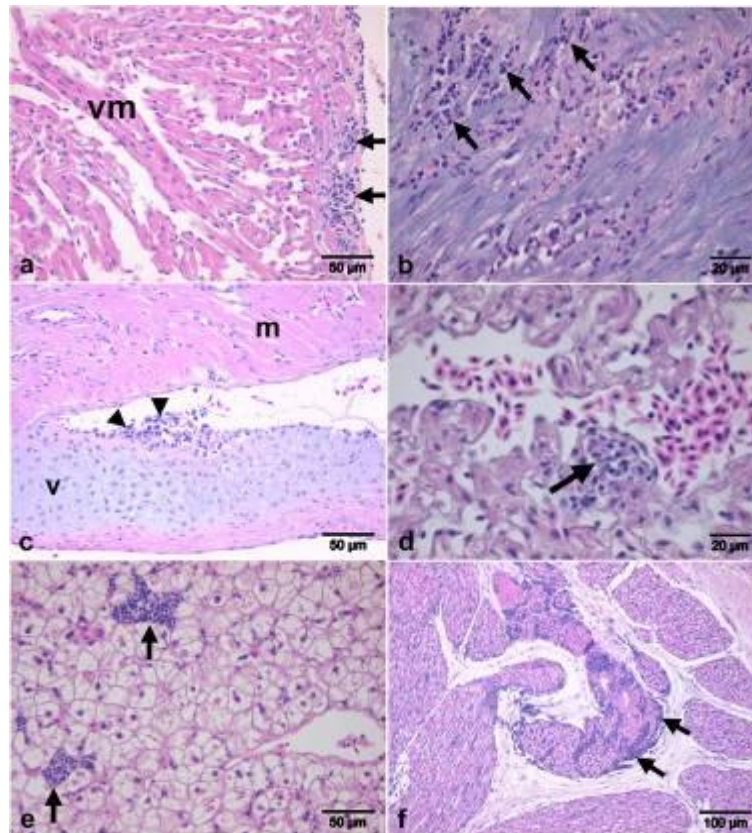


**Figure 36.** Micrographs of histological sections of the heart, liver and swim bladder from a juvenile grouper *E. lanceolatus*, from the oral challenge trial (control group, replicate 2). (**a**) heart with focal pericarditis (arrows), vm= ventricular cardiac muscle (X400) H&E; (**b**) Focal myocarditis of bulbus arteriosus (X400) H&E; (**c**) Focal endocarditis (arrowheads), v=AV, valve m= cardiac muscle (X400) H&E; (**d**) Focal myocarditis of atrium (X1000) H&E; (**e**) liver with hepatitis, note multiple foci of mononuclear inflammatory cells (arrows) (X400) H&E; (**f**) swim bladder with inflammation of the rete mirabile (arrows) (X200) H&E.

**Low Dose**
No Gram-Glynn-positive cocci were visualised by examination of Gram-Glynn special stains of organs and tissues from six fish from the low dose treatment group (Appendix 7). However, there was mild pathology changes observed in many fish, suggesting fish were responding to the presence of a pathogen. Six fish had a very mild branchitiis, one fish had a very mild pericarditis, and three fish had endocarditis, with infiltration of lymphocytic inflammatory cells in the endocardium of the atrio-ventricular valves (Fig. 37a, b & c). A few fish had myocarditis (Fig. 37c), with swelling and vacuolation of the ventricular myocardium

and endothelium (Fig. d). One fish (fish 4) had mild focal necrosis of kidney tubule epithelial cells (Fig. 37e). This fish also had phlebitis, with an increased number of circulating inflammatory cells (Fig. 37f). One fish had a mild granulomatous inflammatory reaction in the haematopoeitic tissue of the kidney (Fig. 37e), but no bacteria were observed by examination of Gram-Glynn stain. The livers of all six fish examined had good lipid storage with well-vacuolated hepatocytes, and a very mild hepatitis, with multifocal aggregations of mononuclear inflammatory cells around small blood vessels.

**High Dose**
Histopathology examination of six fish from the high dose treatment group, showed three of six fish examined were infected with *S. agalactiae*. Gram-Glynn-positive cocci, were detected, by examination of Gram-Glynn stain, variously, in the brain, heart, spleen or gills (Appendix 7).

All six fish had a mild branchiitis (as described already). One fish had marked eye pathology, with a prolific granulomatous inflammatory response in the choroid space (Fig. 38a). Multiple granuloma-like lesions were dispersed throughout the choroid space, the granulomas containing a central core consisting mainly of macrophages full of Gram-Glynn-positive cocci, (Fig. 38f). In contrast, the eyes of two fish had a mild choroiditis and iritis.

The brain of one fish had marked meningitis with granulomas adhered to the meninges. Two fish had mild meningitis, with *S. agalactiae* confirmed by the presence of Gram-Glynn-positive cocci in macrophages circulating in the blood vessels of the meninges (Fig. 38e).

One fish had a severe peritonitis, with prolific granulomatous inflammatory response in the peritoneal cavity. The other five fish had no observable changes of the peritoneal cavity, and there was little pathology observed in the stomach, intestines, pancreas, skin or skeletal muscle of all fish examined.

All six fish in this treatment group had changes in the heart. Three fish had pericarditis, ranging from mild to moderate (fish 4 & 5) to severe (fish 1). The pericarditis was characterized by a lymphocytic inflammatory response in the pericardium of the ventricle, atrium or bulbus arteriosus (Fig. 38b). Two fish (fish 3 & 6) had focal myocarditis with a mixed granulocytic and lymphocytic inflammatory infiltration of the myocardium. Two fish had a prominent endocarditis of the atrio-ventricular valves, but no bacteria were visualised on the valves from examination of Gram-Glynn stain.

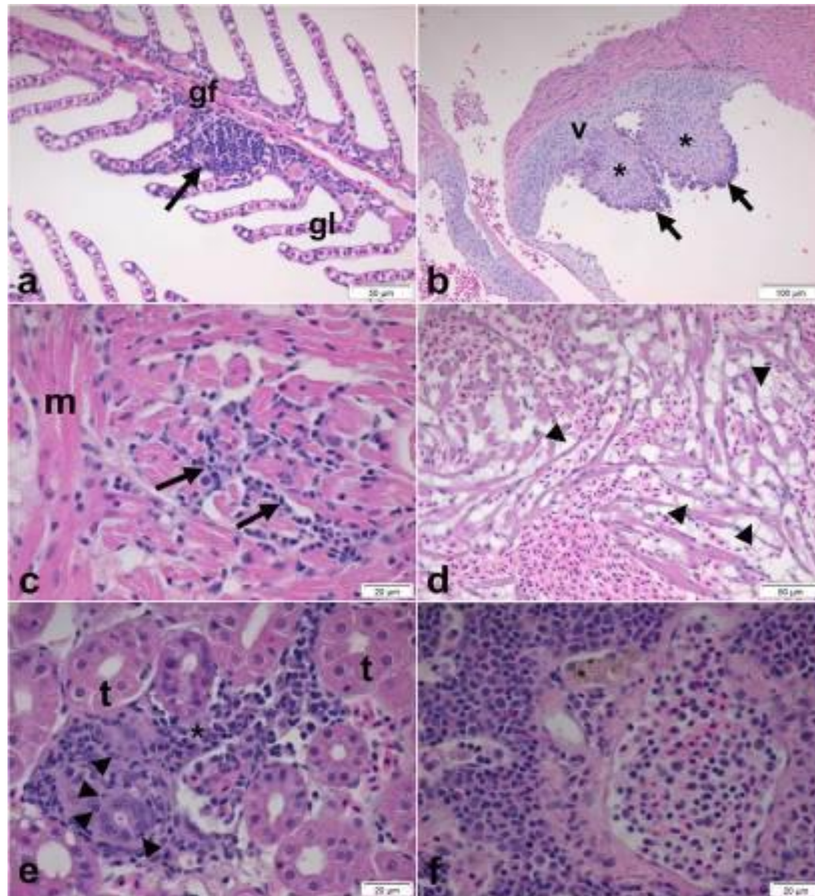


**Figure 37.** Micrographs of histological sections of the gills, heart, and caudal kidney from a juvenile grouper *E. lanceolatus*, from the oral challenge trial (low dose, replicate 2). (**a**) gills with rare foci of inflammatory cells (arrow) between adjacent gill lamellae (gl), gf=gill filament, (X400) H&E; (**b**) heart valve with prolific endocarditis (*, arrows), v= valve (X200) H&E; (**c**) Heart ventricle with focal myocarditis (arrows) (X400) H&E; (c) Focal myocarditis (arrows), m = cardiac muscle (X400) H&E; (**d**) ventricular cardiac muscle with swollen, vacuolated endocardium (arrowheads) (X1000) H&E; (**e**) caudal kidney with focal necrosis of kidney tubule cells and scattered necrosis of haematopoietic tissue (arrowheads, *) t = kidney tubule (X1000) H&E; (**f**) head kidney with phlebitis. Note melano-macrophage adjacent to blood vessel (X1000) H&E.

The caudal kidneys of most fish in the high dose treatment group, had a mild nephrosis with infiltrates of lymphocytes into the haematopoietic tissue. One fish (fish 2) had some necrosis of blood vessel walls and tubule epithelium, with an increased number of eosinophilic granular cells and lymphocytes among the haematopoeitic tissue. One fish (fish 4) had several kidney tubules, with lumens filled with a faintly basophilic, cast-like material (as described earlier) and with several large melano-macrophages among the haematopoietic tissue. The spleens of all fish were congested, with reduced white pulp and with melanin pigment dispersed and scattered throughout the red and white pulp, but especially prominent around the ellipsoids and larger vessels (Fig. 38c). There were also local accumulations of melanin and melano-macrophages within the walls of splenic veins and arteries (Fig. 38c). The livers of five fish had a mild hepatitis, with perivascular inflammation and aggregations of mononuclear inflammatory cells in the hepatic parenchyma. There was also cholangiohepatitis in a few fish, with mixed, but mainly mononuclear inflammatory infiltrate surrounding the bile ducts (Fig. 38d).

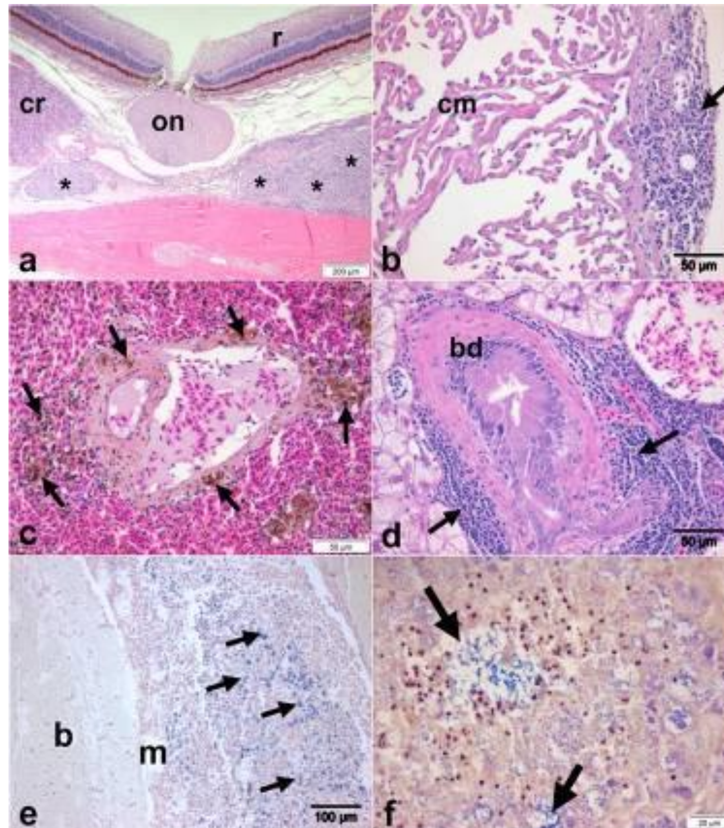


**Figure 38**. Micrographs of histological sections of the eye, heart, spleen, caudal kidney and brain from a juvenile grouper *E. lanceolatus*, from the oral challenge trial (high dose, replicate 2). (**a**) eye. Note granulomas (*) in the choroid space and adjacent to choroid rete (cr) (arrow) r = retina, on = optic nerve (X100) H&E; (**b**) heart ventricle with pericarditis. Note inflammation (arrow), cm = cardiac muscle (X400) H&E; (**c**) spleen with melanin in pulp and in wall of blood vessel (arrows) (X400) H&E; (**d**) liver with cholangiohepatitis. Note inflammation (arrows) of bile duct (bd) (X400) H&E; (**e**) brain with meningitis. Note the thick granulomatous inflammatory infiltrate, and blue-staining *S. agalactiae* in macrophages (arrows) (X200) Gram Glynn; (**f**) higher power view of a granuloma in choroid. Note blue-staining *S. agalactiae* in macrophages (arrows), among inflammatory cells (X1000) Gram Glynn.

### Statistical comparison of test results & discussion

In the oral challenge trial, (for both replicates), detection rates of *S. agalactiae*, from bacteriology, PCR and histology tests differed. Comparison of results of PCR assay with bacterial isolation and histology (for pooled organ test results) showed variability in test results, depending on the test (Table 27). For example, some fish tested negative by bacteriology, but tested positive by PCR, or vice versa. The numbers of fish testing positive by each test method, compared to another test method are shown in Tables 27 & 28 (below). McNemar tests were used to compare detection rate differences.

**Table 27.** Results for the oral challenge trial, replicate 1, for each fish (pooled organs), from PCR, histology and bacteriology.

| | | **PCR** | | |
|---|---|---|---|---|
| | | + | **-** | **Total** |
| **Histology** | + | 3 | 1 | **4** |
| | **-** | 19 | 0 | **19** |
| | **Total** | **22** | **1** | **23** |

| | | **PCR** | | |
|---|---|---|---|---|
| | | + | **-** | **Total** |
| **Bacteriology** | + | 1 | 1 | **2** |
| | **-** | 21 | 0 | **21** |
| | **Total** | **22** | **1** | **23** |

| | | **Histology** | | |
|---|---|---|---|---|
| | | + | **-** | |
| **Bacteriology** | + | 1 | 1 | **2** |
| | **-** | 3 | 18 | **21** |
| | **Total** | **4** | **19** | **23** |

**Table 28.** Results for the oral challenge trial, replicate 2, for each fish (pooled organs), from PCR, histology and bacteriology.

| | | **PCR** | | |
|---|---|---|---|---|
| | | + | **-** | **Total** |
| **Histology** | + | 3 | 0 | **3** |
| | **-** | 9 | 1 | **10** |
| | **Total** | **12** | **1** | **13** |

| | | **PCR** | | |
|---|---|---|---|---|
| | | + | **-** | **Total** |
| **Bacteriology** | + | 1 | 1 | **2** |
| | **-** | 11 | 0 | **11** |
| | **Total** | **12** | **1** | **13** |

| | | **Histology** | | |
|---|---|---|---|---|
| | | + | **-** | |
| **Bacteriology** | + | 0 | 2 | **2** |
| | **-** | 3 | 8 | **11** |
| | **Total** | **3** | **10** | **13** |

For replicate 1, comparison of test results using the McNemar test showed that PCR was significantly different ($p < 0.05$) from both histology and bacteriology, indicating PCR is a more sensitive test for detecting *S. agalactiae*. Histology and bacteriology were not significantly different (Table 29).

The results from replicate 2 confirm the findings of replicate 1, with the same statistical differences between the three test methods. PCR is a more sensitive test for detecting *S. agalactiae* that histology or bacteriology (Table 30).

**Table 29.** Statistical values for comparison of laboratory test results (PCR, histology and bacteriology) for fish from the oral challenge trial, replicate 1, using McNemar Test.

| **Replicate 1 test comparison** | |
|---|---|
| **Test** | **% positive** |
| PCR | 95.7a* |
| Histology | 17.4b |
| Bacteriology | 8.7b |

*percentages followed by a similar letter do not differ significantly ($p>0.05$)

**Table 30.** Statistical values for comparison of laboratory test results (PCR, histology and bacteriology) for fish from the oral challenge trial, replicate 2, using McNemar Test.

| **Replicate 2 test comparison** | |
|---|---|
| **Test** | **% positive** |
| PCR | 92.3a* |
| Histology | 23.1b |
| Bacteriology | 15.4b |

*percentages followed by a similar letter do not differ significantly ($p > 0.05$)

Fish from the unchallenged controls showed 100% concordance, ie. all were negative by PCR, bacteriology and histology tests.

**Discussion of statistical test results**

***PCR and bacteriology***

The discrepancy between results from bacterial culture and PCR may be explained by the possibility of sampling or human error in methodology used, for either test method. For example, for bacteriology human error can occur when sampling from whole fish tissues, from cross-contamination between samples, during bacterial swabbing and culture, leading to a false negative or positive result. Sampling error may occur in that by chance the sample taken is not representative. This may have occurred in one case (fish 1, replicate 2, high dose treatment group), where the fish was negative for *S. agalactiae* from bacteriology done on the brain, kidney spleen, but the fish showed clinical signs of infection (was moribund before euthanased), was PCR positive (brain) and was positive by histology, showing severe pericarditis, meningitis, hepatic necrosis, choroiditis, nephritis. Gram-Glynn-positive cocci visualised from the brain, peritoneal cavity, and heart, confirming this fish was infected with *S. agalactiae*. Alternatively, there may be a low sensitivity of detection by bacteriology on blood agar for *S. agalactiae*, however this is unlikely given that bacteriology is considered the Gold Standard Test for isolation of marine bacteria from fish.

Another possibility is that many surviving, healthy fish, that tested positive by PCR, were subclinical carriers of *S. agalactiae*, ie. they overcame the infection (and hence showed no clinical signs of infection), as the PCR was picking up bacterial DNA that had been phagocytosed by macrophages. Experimental observations support this hypothesis, since although most fish initially showed clinical signs of infection (becoming anorexic and lethargic) early on in the experiment, after 2.5 weeks the fish regained their appetites, and appeared healthy, with 74 of the total 76 fish surviving the five weeks duration of the challenge trial, showing no overt clinical signs of infection. Only two fish appeared moribund and displayed clinical signs of infection. Both these fish were euthanased, tested PCR positive, histology positive, and one was positive by bacterial analysis (Appendix 7).

In the oral challenge trial, of the fish that were tested by all 3 test methods, 23 of the 24 surviving healthy fish (fed either low or high doses of *S. agalactiae*) tested PCR positive; seven of these fish also tested positive by histology; two of these were also positive by bacteriology and two of these fish were positive by all three test methods. It is therefore most likely that PCR is the most *sensitive* test method for detecting *S. agalactiae*, and this is supported by statistical analyses. PCR is able to detect persistent bacterial DNA from dead or phagocytosed *S. agalactiae.* Therefore subclinical carriers (ie. healthy fish), may test positive by PCR, but negative by histology analysis and bacterial isolation.

***PCR and Histopathology***
The discrepancy between results from PCR and histopathology may have resulted from several factors. Human error may have occurred during histological interpretation of tissue, whereby bacteria were not detected with the naked eye, especially if in low numbers, leading to a false negative result. This is possible since slides were examined for 20 minutes each and searched thoroughly for bacteria, after which a negative was recorded if no bacteria were detected. Alternatively, the tissue section may not have had any bacteria present, since histology slides contain only one small slice of a whole organ, therefore leading to a false negative (test limitation).

For test results where histopathology was negative and PCR was positive, the most likely explanation is that surviving fish were subclinical carriers, and PCR was detecting DNA from dead or phagocytosed *S. agalactiae.* This is supported by experimental mortality results, where all fish (except two) survived the entire five weeks duration of the experiment. Alternatively, fish may have just becoming infected with the bacteria, and may have eventually developed the infection and showed clinical signs of infection, if the duration of the experiment had extended, however this is unknown, since the experiment was terminated after five weeks.

***Histopathology and Bacteriology***
For test results where histopathology was positive and bacteriology was negative, the most likely explanation is human error or low sensitivity of detection by bacterial culture.

In conclusion, PCR was consistently the most sensitive test for detecting *S. agalactiae* from the brain, heart or spleen of fish sampled in both the oral challenge trials. It must be noted that PCR will detect bacterial DNA, such that a positive fish may represent either a clinically infected fish, or a sub-clinical carrier of *S. agalactiae* (ie. the fish has become infected, overcome the infection, and survived, showing no clinical signs of infection). Therefore, PCR together with at least one other test method (such as histology and/or bacteriology) are recommended to be most useful for diagnosing and confirming *S. agalactiae* infection in fish.

**Experiment 5: cohabitation challenge trial**
***Clinical signs***
In the cohabitation challenge, within the first three days, large numbers of fish stopped feeding and stayed at the bottom of their tank with little reaction to external stimuli. These fish were mainly injected animals with a few non-injected fish also affected. In replicate 1, the number of fish remaining at the bottom within the first ten days was fourteen, five and two respectively for the 1:1, 1:2 and 1:5 ratios. In replicate 2, the number of fish remaining at the bottom within the first ten days was respectively ten, eight and three. Fish returned to feeding over time, with all fish apparently healthy by two and a half weeks post challenge. In total, only two fish in each system died; both were injected cohabitant fish, (one fish from ratio 1:5 and one fish from ratio 1:1). However, the number of lethargic fish staying at the bottom of the tank, without feeding, decreased over time, suggesting that these animals were successfully overcoming or clearing the infection.

Juvenile grouper that showed clinical signs of infection with *S. agalactiae* were removed from tanks and euthanased. Only two fish developed exophthalmia; both were injected (ie. cohabitant fish), from the 1:1 ratio (one per replicate). Two fish displayed erratic swimming; both were injected fish, (from replicate 2); ratio 1:5 and 1:2 (Appendices 8 & 9). A few fish had gross clinical signs of infection, including hyperaemia and swollen meninges, ascites,

splenomegally or an enlarged kidney, but no red skin lesions or dermal oedema were observed.

This result of low mortalities in the injected cohabitants, is contradictory to the results obtained in the injection challenge trial, where high fish mortalities were observed in fish injected with the same dose (ie. $10^6$ cfu fish$^{-1}$). There are several possible explanations for the differing results. One possible explanation is that a loss of virulence in the bacterial strain may have occurred through repeated subculture. However, in this case, a master-seed stock was used, (in line with best practice), and there was no repeated subculture of the strain, so this seems an unlikely explanation. A second possible explanation is genetic variation between different batches of grouper used in the different experimental challenge trials. The fish used in the oral and cohabitation challenges were of a different genetic stock/lineage (with different broodstock parents) to fish used previously in the injection and immersion challenge trials. Fish used in the oral and cohabitation trials came from new batches of larvae, reared from newly introduced wild *E. lanceolatus* broodstock grouper that were subsequently spawned at NFC. It is possible fish with a differing genetic/immune background may have responded with different immunogenetic variability, to the same pathogenic strain of *S. agalactiae* to that used in previous experimental challenge trials. A third explanation is that the husbandry conditions of both recirculating aquarium systems differed. The systems were slightly modified and improved between trials, by the addition of a protein skimmer (foam fractionation) on each system. This resulted in significantly improved water quality, by removing excess organic material, including proteins and amino-acids, before they broke down into nitrogenous wastes. Mortalities in replicate system 2 were also higher in number than those in replicate system 1. These results were similar to those obtained in the immersion challenge trial. These differences were attributed to a measured decrease of the water quality in replicate system 2. Poor water quality is known to have a deleterious effect on fish health status and therefore the improvements in the systems for this challenge may explain the low level of mortality. Ammonia levels throughout the duration of the cohabitation and oral challenges were below the detectable limit of the kits used.

***PCR***

The PCR results (pooled PCR results from individual whole organs) from non-injected fish, varied between treatment groups (ie. different ratios) of cohabitant (injected fish): cohabittee (non-injected fish). In replicate 1, 100% of fish in ratio 1:5 tested positive, 83% of fish in ratio 1:2 tested positive, and 60% of fish in ratio 1:1 tested positive for *S. agalactiae* by PCR (Table 31). In comparison, in replicate 2, 83% of fish in ratio 1:5 tested positive, 50% of fish in ratio 1:2 tested positive, and 30% of fish in ratio 1:1 tested positive for *S. agalactiae* by PCR (Table 31). In the control groups, 33% (two of six fish tested) were positive for *S. agalactiae* by PCR in replicate 1, and one fish was positive for *S. agalactiae* by PCR in replicate 2.

***Bacteriology***

Bacteriology results varied between different treatment groups, and between different replicate systems. In replicate 1, *S. agalactiae* was recovered by bacteriology from; zero of eight fish tested (0%) from ratio 1:5; two out of thirteen fish tested (15%) from ratio 1:2 (one non-injected fish, one injected fish) and seven out of nineteen fish tested (37%) from ratio 1:1 (six injected fish, one non-injected fish (Appendix 8).

In replicate 2, *S. agalactiae* was detected by bacteriology in; one of eight fish tested (12.5%) from ratio 1:5 (1 injected fish); two of eleven fish tested (18%) from ratio 1:2 (two injected fish; and six of 20 fish tested (30%) from ratio 1:1 (Appendix 9).

***Histopathology summary***
Histopathology results varied, both between different treatment groups and between different replicate systems. These results are for whole fish analysis.

In replicate 1; four of six fish (66.6%) from ratio 1:5 (one of two injected fish, three of six non-injected); five of eleven fish (45%) from ratio 1:2 (4 of 5 injected fish; one of six non-injected fish); nine of nineteen fish (47%) from ratio 1:1 (nine injected fish, zero non-injected fish) tested positive for *S. agalactiae* by histopathology analysis (Appendix 8). All control fish tested negative by histopathology. Overall combined results for this replicate, showed a total of eighteen fish, of a total of 44 fish tested (41%), were positive by histopathology.

In replicate 2, six of eight fish 75% from ratio 1:5 (two of two injected fish, four of six non-injected fish); five of eleven fish (45%) from ratio 1:2 (four of five injected fish, one of six non-injected fish); and ten of 20 fish (50%) from ratio 1:1 (ten of ten injected fish, zero non-injected fish) tested positive for *S. agalactiae* by histopathology analysis. One of six fish from the contol group also tested positive (Appendix 9). Overall combined results for this replicate, showed a total of 22 fish, of a total of 45 fish tested (49%), were positive by histopathology.

***Laboratory test results summary***
Laboratory test results including PCR, bacteriology and histopathology, indicated that healthy juvenile grouper *E. lanceolatus*, can become infected with *S. agalactiae,* by cohabitation, of infected fish (fish injected with *S agalactiae*) with non-infected fish, (cohabitee ie. healthy, non-injected fish) at ratios of 1:1, 1:2 and 1:5 (Table 31). A few fish in each of the control treatment groups, (both replicate systems 1 & 2), also became infected with *S. agalactiae*, indicating *S. agalactiae* is transmissable in water between individual tanks, (despite physical separation of fish cohorts and of different treatment groups), and despite good biological filtration. This is not surprising, since no ozone treatment of water was done, and because experiments were done in a recirculation system (not ideal). These results give further evidence that *S. agalactiae* can be transmitted by water, from infected to uninfected fish, (by cohabitation of infected and non-infected fish) within a tank, and between tanks, ie. from tank to tank in a recirculation system, (despite physical separation of tanks and good biological filtration with a protein skimmer).

Detection of *S. agalactiae* in the various organs and tissues from each fish, showed variable results, depending on the test used (see Appendices 8 & 9 for detailed results). Because of this, statistical analyses using McNemar's test was used to compare the various test results for each treatment group within each replicate system. Statistical analyses indicated PCR was the most sensitive test for detecting *S. agalactiae* DNA in fish organs and tissues, compared to bacteriology and histology.

**Table 31. Combined laboratory results (PCR, bacteriology and histopathology) for the cohabitation challenge trial.** C1=Cohabitation ratio 1:5 (2 injected:10 non-injected fish) (n=12); C2=Cohabitation ratio 1:2 (5 injected:10 non-injected fish) (n=15); C3=Cohabitation Ratio1:1 (10 injected:10 non-injected fish), (n=20), *one fish only (other found dead, histology uninterpretable). #sample missing so not analysed. Number in brackets denotes number of fish analysed by each test, for each treatment group. Number in each cell denotes a positive result.

| Treatment Group | Replicate 1 | | Replicate 2 | |
|---|---|---|---|---|
| **C1 (Ratio 1:5)** **Test:** | **Injected (n=2)** | **Non-injected (n=6)** | **Injected (n=2)** | **Non-injected (n=6)** |
| PCR | 2 | 6 | 2 | 5 |
| Bacteriology | 0 | 0 | 1 | 0 |
| Histology | 1* | 3 | 2 | 4 |
| **C2 (Ratio 1:2)** **Test:** | **Injected (n=5)** | **Non-injected (n=6)** | **Injected (n=5)** | **Non-injected (n=6)** |
| PCR | 5 | 5 | 4 | 3 |
| Bacteriology | 1 | 1 | 2 | 0 |
| Histology | 4 | 1 | 4 | 1 |
| **C3 (Ratio 1:1)** **Test:** | **Injected (n=9)** | **Non-injected (n=10)** | **Injected (n=10)** | **Non-injected (n=10)** |
| PCR | 9# | 6 | 10 | 3 |
| Bacteriology | 6 | 1 | 5 | 1 |
| Histology | 9 | 0 | 10 | 0 |
| **Control** **Test:** | | **Non-injected (n=6)** | | **Non-injected (n=6)** |
| PCR | - | 2 | - | 1 |
| Bacteriology | - | 0 | - | 0 |
| Histology | - | 0 | - | 1 |

**Histopathology interpretation of juvenile Queensland grouper examined from Experiment 5, the cohabitation challenge trial (replicate 1)**

**Ratio 1:1 (ten injected cohabitant fish: ten non-injected cohabittee fish)**

***Injected cohabitant fish (9 fish examined)***
Histopathology examination of nine fish from this treatment group showed all were positive for *S. agalactiae*. Gram-Glynn-positive cocci were visualised in multiple organs from all nine fish, from examination of Gram Glynn special stains. All fish had marked pathology in multiple organs and tissues, similar to that observed in the injection trial (as previously described in Section 6.6).

***Non-injected cohabittee fish (ten fish examined)***
Histopathology examination of nine fish from this treatment group showed all were negative for *S. agalactiae*. No bacteria were visualised from detailed examination of all organs and tissues stained with Gram-Glynn stain. A few fish had very mild branchitis, a very mild choroiditis and mild hepatitis (as described previously). The skin of most fish had a mild subdermal oedema. One fish had enteritis, but no bacteria were associated with this lesion. One fish had a mild inflammation of the swimbladder with infiltrations of mononuclear cells into the connective tissue of the rete mirabile, gas gland secretory cells, and with scattered cellular necrosis of rete mirabile connective tissue, but no bacteria were detected in association with the inflammation.

**Ratio 1:2 (six injected cohabitant fish: twelve non-injected cohabittee fish)**

***Injected cohabitant fish (five fish examined)***
Histopathology examination of five fish from this treatment group, showed four of five fish were positive for *S. agalactiae* (fish 1, 2, 3, 4, Appendix 8). Pathology was marked, with Gram-Glynn-positive cocci visualised in multiple organs and tissues (as described in section 6.6). There was marked granulomatous meningitis, with large granulomas extending out from the meninges into the ventricles (Fig 39a, b). The granulomas were well-circumscribed, with central cores consisting of aggregations of Gram-Glynn-positive cocci, clearly visible in enlarged macrophages, staining positive with Gram Glynn special stains (Fig. 39e, f). Eye lesions included a prolific choroiditis, with multiple granulomas in the choroid space, corneal stromal oedema and malacia, especially in the limbic region, often extending bi-directionally along the corneal stromal layers (Fig 39a, b, c & d). One fish (fish five) had mild iritis, branchitis, enteritis, and the spleen had numerous melano-macrophages dispersed throughout.

***Non-injected cohabittee fish (six fish examined)***
Histopathology examination of six fish from this treatment group showed only one of six fish was positive for *S. agalactiae* (fish 1, Appendix 8). Eye pathology included severe ophthalmitis, with inflammation and oedema of the epi-scleral collagen layers of the peri-orbital connective tissues. There was keratitis characterised by abnormal corneal thickening, neo-vascularisation and inflammation of the corneal stromal layers, and periorbital inflammation and myofasciitis (Fig. 40a, b). Gram-Glynn-positive cocci were visible in the inflammatory infiltrate (Fig. 40b). There was meningitis, skeletal muscle fasciitis, hydropic degeneration of the liver and peritonitis. This fish had marked pericarditis of the bulbus arteriosus, ventricle and atrium (Fig. 40c, d & e). The spleen was congested with massive granulomatous inflammation. The gills had a mild branchitis. The remaining five fish had some very mild pathology; one fish (fish 5) had a mild scleritis and iritis; four fish (fish 2, 3,

4 and 5) had a mild pericarditis of the bulbus arteriosus, ventricle or atrium. The spleen, head and caudal kidney of all fish, had low to moderate numbers of large melano-macrophages scattered throughout the organs (Fig. 39f).

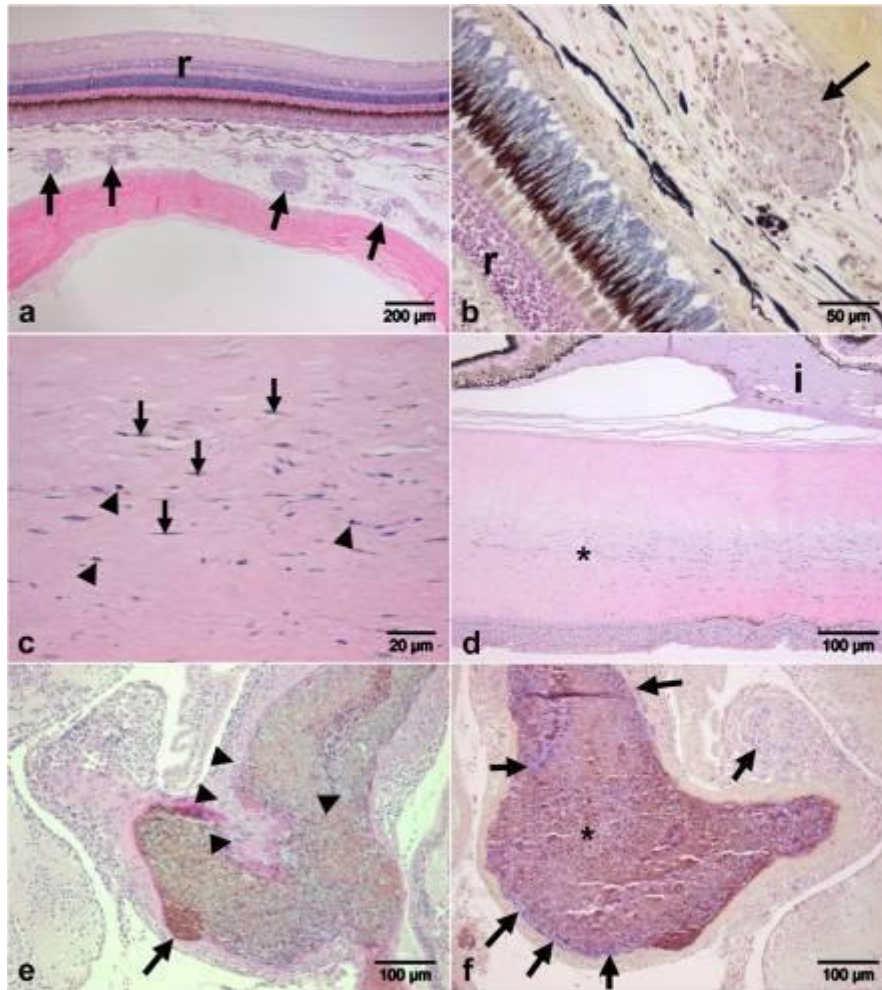


**Figure 39**. Micrographs of histological sections of the eye and brain from juvenile grouper E. lanceolatus, from the cohabitation challenge trial (replicate 1, injected cohabitant fish, ratio 1:2). (**a**) eye with multiple granulomas in the choroid space (arrows), r= retina (X100) H&E; (**b**) higher power view of a granuloma (arrow) containing *S.agalactiae* bacteria (X400) Gram Glynn; (**c**) eye, corneal stromal oedema and malacia, with necrotic cells (arrowheads) (X1000) H&E; (**d**) eye cornea with corneal oedema and malacia in limbic region (*), i=iris (X200) H&E; (**e**) large bacterial granuloma (arrow) in brain ventricle containing macrophages full of bacteria (arrowheads) (X200) H&E; (**f**) large brain granuloma (*) contains multiple macropahges with blue-staining *S.agalactiae* bacteria (arrows), also visible in meninges (X200) Gram Glynn.

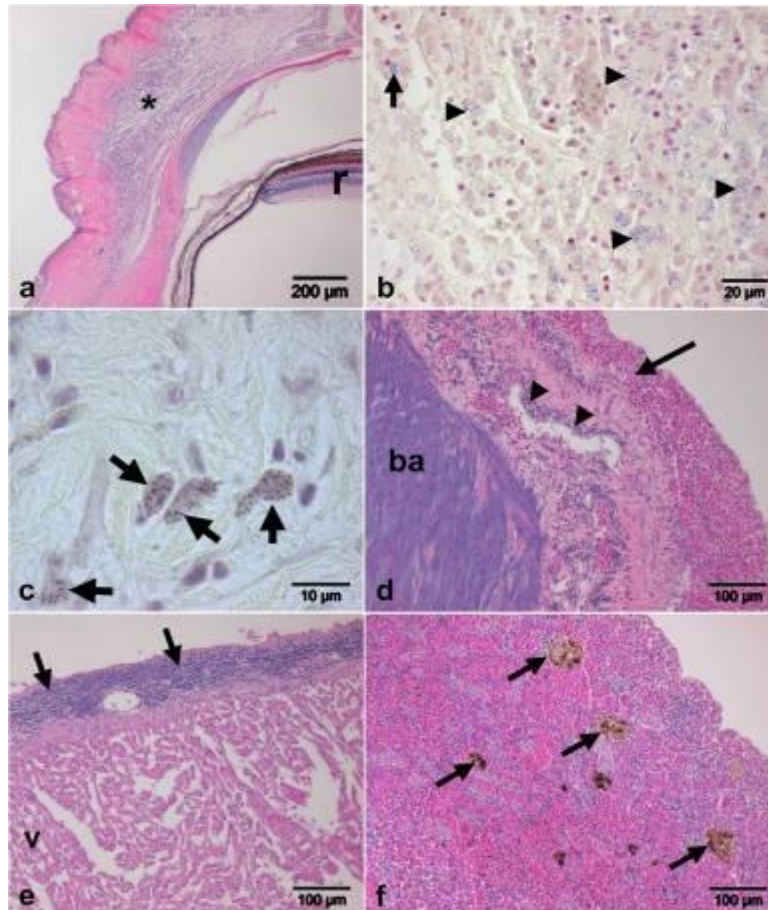


**Figure 40.** Micrographs of histological sections of the eye, heart and spleen from juvenile grouper *E. lanceolatus*, from the cohabitation challenge trial (replicate 1, ratio 1:2, non-injected cohabitee fish). (**a**) eye with inflammation (*) of periorbital skeletal muscle and connective tissues (X100) H&E; (**b**) higher power view of (a) showing mixed inflammatory infiltrate, and macrophages containing blue-staining *S.agalactiae* bacteria (X1000) Gram Glynn; (**c**) Gram-Glynn-positive cocci in macrophages of the bulbus arteriosus (arrows) (X1000) Gram Glynn; (**d**) bulbus arteriosus with perivascular inflammation of coronary vessels (arrowheads) and haemorrhage (long arrow) (X200) H&E; (**e**) heart ventricle. Note marked pericarditis (arrows) (X200) H&E; (**f**) spleen with multiple melano-macrophage centres (arrows) (X200) H&E.

### Ratio 1:5 (2 injected cohabitant fish: six non-injected cohabittee fish)

***Injected cohabitant fish (two fish examined)***

One of the two fish examined had extensive post mortem degeneration, such that histological interpretation of tissue changes was not possible. The other had histopathology consistent with infection with *S. agalactiae.* (as described previously for injected fish from, Section 6.6).

***Non-injected cohabittee fish (six fish examined)***

Histopathology examination of six fish from this treatment group showed three of six fish examined were positive for *S. agalactiae*. Bacteria were visualised in the brain meninges in all three fish, and in the choroid of one of these fish (Appendix 8). All fish variably, had a mild choroiditis, iritis, enteritis, hepatitis, peritonitis, pericarditis, endocarditis, and myocarditis of the ventricle, atrium and bulbus arteriosus. The spleen in all fish had a large amount of melanin pigment scattered throughout the organ, with many melano-macrophages, indicating possible engulfment and killing of bacteria by macrophages. Although no bacteria

were visualised in the other three fish by histopathology analysis, these three fish had mild pathology including; dermatitis, myositis, hepatitis, choroiditis, iritis, peritonitis, pericarditis, endocarditis. Interestingly, these fish were positive by PCR.

**Replicate 2**
**Ratio 1:1 (ten injected cohabitant fish: ten uninjected cohabittee fish)**

***Injected cohabitant fish (ten fish examined)***
Examination of ten cohabitant fish from this treatment group showed all were positive for *S. agalactiae*, with Gram-Glynn-positive cocci visualised in multiple organs of all fish stained with Gram-Glynn stain. There was variable but marked pathology observed in all fish examined (as described earlier for injected fish, Section 6.6).

***Non-injected cohabittee fish (ten fish examined)***
Histopathology examination of ten fish from this treatment group showed all were negative for *S. agalactiae*. A few fish had very mild pathology including mild branchitis, (Fig. 41b). One fish (fish 6) had a deformed and reduced size swimbladder with hypertrophy of the gas gland epithelial secretory cells, the cell nuclei hypertrophied and with dispersed and marginated chromatin (Fig. 41a).This fish also had a reduced size eye, with retinal dysplasia, the retinal layers deformed and with abnormal folding. A few fish had a mild dermatitis. The spleens and livers were as described earlier. Three fish (fish 6, 7 & 8) had unidentified microsporidian-like parasites in the spleen, head and caudal kidney. Multiple (presumable) sporophorous vesicles, containing spores, were detected among the haematopoietic tissue of the head and caudal kidney and in the spleen and liver, but there was little inflammatory response associated with this parasite (Fig. 41c, d, e, f). Similar undescribed microsporidian-like parasites have been detected in numerous reef fish species examined by histopathology by the project leader, in routine diagnostic examination of fish from the Great Barrier Reef region in previous years.

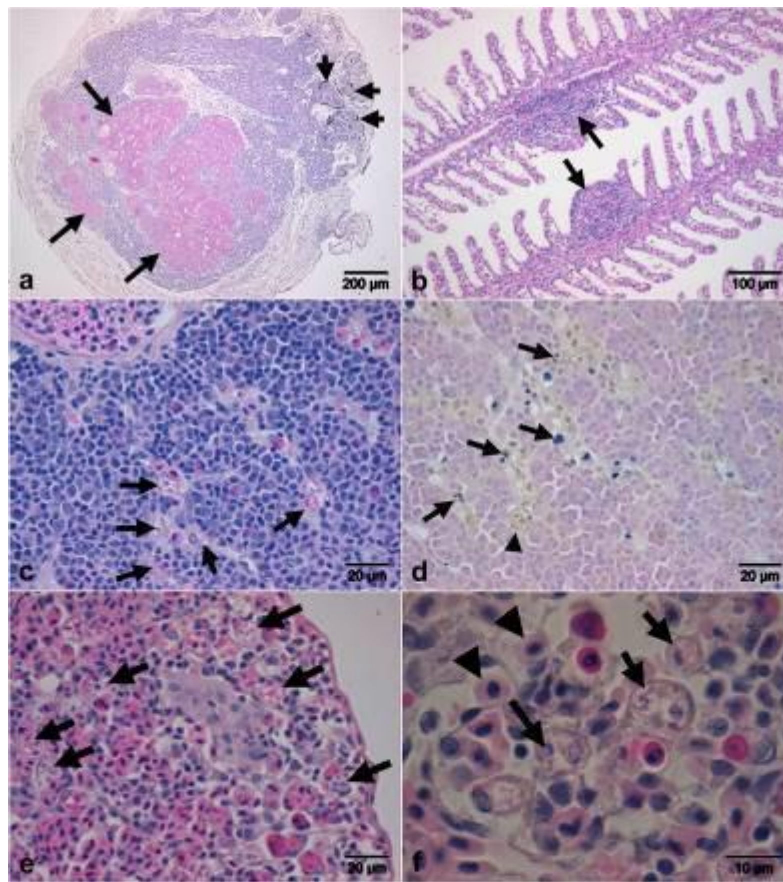


**Figure 41**. Micrographs of histological sections of the swim bladder, gills, head kidney and spleen from juvenile grouper *E. lanceolatus*, from the cohabitation challenge trial (replicate 2, ratio 1:1, non-injected cohabitee fish) (**a**) Abnormal, reduced size swim bladder with hypertrophy of the gas gland epithelial secretory (arrows, lower left) and melanin in rete mirabile (arrows, upper right) (X100) H&E; (**b**) Gills with multiple foci of mixed inflammatory infiltrate (arrows) (X200) H&E; (**c**) head kidney with microsporidean-like parasites (arrows) (X400) H&E; (**d**) Giemsa stain showing blue-staining parasites (arrows) and a sporophorous vesicle with spores (arrowhead) (X400) Giemsa; (**e**) Spleen infected with microsporidian-like parasites (arrows) (X400) H&E; (**f**) higher power view of (e), spleen, with multiple presumable, microsporidian sporophorous vesicles containing spores (arrows) arrowheads=host erythrocytes (X1000) H&E.

**Ratio 1:2 (six injected cohabitant fish: twelve non-injected cohabittee fish)**

***Injected cohabitant fish***
Histopathology examination of five fish from this treatment group showed four fish (fish 1, 2, 3 & 4) were positive for *S. agalactiae.* Variable pathology included meningitis, ophthalmitis, branchiitis, pericarditis, myocarditis, hepatitis, splenitis, nephritis, haemorrhagic enteritis, gastritis, serositis, peritonitis, dermatitis, skeletal muscle degeneration and fasciitis, mesenteric endarteritis and vasculitis, with pathology as described for injected fish from section 6.6. One fish had endocarditis.

Four fish (fish 1, 2, 3 & 4) had meningitis, the meninges thickened with a mixed inflammatory infiltrate and with macrophages containing Gram-Glynn-positive cocci. Colonies of Gram-Glynn-positive cocci were also detected in circulating macrophages in the lumen of blood capillaries in the brain. Multiple bacterial granulomas, of moderate to large size, were in the third ventricle, the granulomas containing a central core of Gram-Glynn-positive cocci (pathology described earlier).

Four fish (fish 1, 2, 3 & 4) had ophthalmitis, including a moderate to marked choroiditis, iritis and keratitis. A granulomatous inflammatory infiltrate was present in the choroid rete and choroid space, with intracellular, Gram-Glynn-positive cocci in enlarged macrophages, in the central core of granulomas, or in the choroid space. The iris was similarly inflamed as was the periorbital skeletal muscle and epi-scleral connective tissue, with a granulomatous inflammatory infiltrate. The cornea was thickened, with a mild infiltration of inflammatory cells beginning at the scleral-limbic junction and extending out into the stromal layers of the cornea. There was increased space between adjacent corneal stromal lamellae, and sometimes separation of descements membrane away from corneal stromal lamellar basement membrane.

***Non-injected cohabittee fish***
Histopathology examination of six fish from this treatment group showed one fish (fish 6) was positive for *S. agalactiae*. This fish had mild iritis (Fig. 42f), with a few Gram-Glynn-positive cocci (within macrophages) in the choroid space. The other five fish in this treatment group were negative, with no bacteria detected. However all five fish had mild pathology indicating the fish were responding to the presence of an antigen, possibly indicating early infection with the bacteria. The gills had a very mild branchitis, (as described earlier). The spleens had melanin pigment in the lumen of the blood vessels and in the peri-ellipsoid region. The livers had good storage vacuolation with mild hepatitis (as described earlier). One fish (fish 1) had a mild iritis (Fig. 42e), mild deep stromal keratitis (as described earlier), meningitis and mild skeletal muscle myositis. Two fish (fish 2 & 3) had very mild atrial myocarditis. Three fish (Fish 2 & 4 & 5) had a reduced size, inflamed and deformed swim bladder, with infiltrates of mononuclear inflammatory cells into the connective tissue layers of the rete mirabile (Fig. 42f). The gas gland secretory cells had hypertrophied nuclei, with dispersed, marginated chromatin. One fish (fish 5) had a very mild pericarditis, with a very mild infiltration of mononuclear inflammatory cells at the apex of the ventricle and junction of ventricle to bulbus arteriosus (Fig. 42a). This fish also had myocarditis of the bulbus arteriousus, with several foci of mononuclear inflammatory cells in myoelastic tissue and endocarditis, with a proliferative inflammation of the atrio-ventricular valve (Fig. 42b, c, d).

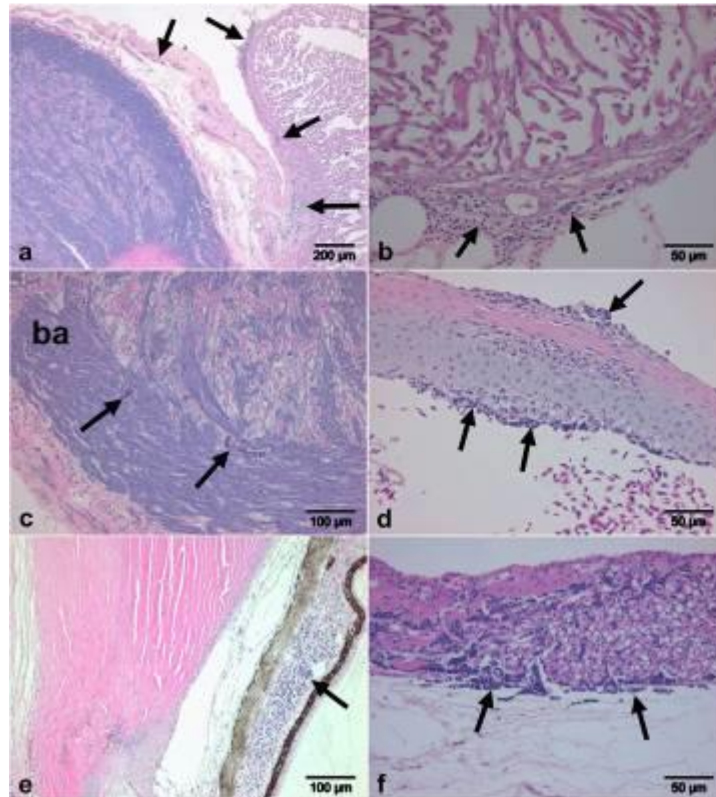


**Figure 42**. Micrographs of histological sections of the heart, eye and swim bladder from juvenile grouper *E. lanceolatus*, from the cohabitation challenge trial (replicate 2, ratio 1:2, non-injected cohabitee fish) (**a**) heart at junction of ventricle and bulbus arteriosus, with pericarditis (arrows) (X100) H&E; (**b**) atria with pericarditis (arrows) (X400) H&E; (**c**) bulbus arteriosus (ba) with multiple inflammatory foci (X200) H&E; (**d**) atrioventricular valve with inflammation (arrows) (X400) (**e**) Eye with inflamed iris (arrow) and oedema at ventral ciliary cleft (to the left) (X200) H&E; (**f**) swim bladder with inflammation of the rete mirabile (arrows) (X400) H&E.

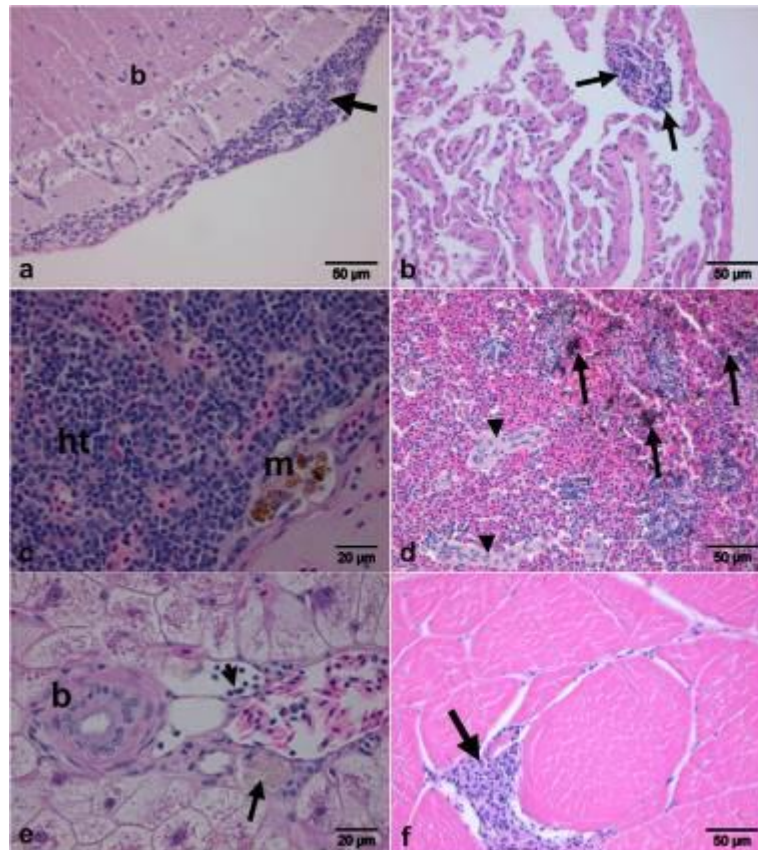


**Figure 43**. Micrographs of histological sections of the brain, heart, head kidney, spleen, liver and skeletal muscle from juvenile grouper *E. lanceolatus*, from the cohabitation challenge trial (replicate 2, ratio 1:2, non-injected cohabitee fish) (**a**) brain (b) with marked meningitis (arrow) (X400) H&E; (**b**) heart atrium with focal myocarditis (arrows) (X400) H&E; (**c**) head kidney with melano-macrophage centre (m) adjacent to blood vessel (X400), ht = haematopoietic tissue H&E; (**d**) spleen with multiple foci of melanin and haemosiderin pigment (arrows), arrowheads = ellipsoids (X400); (**e**) liver with a melano-macrophage centre adjacent to splenic vein . Note inflammatory cells (short arrow) (X1000) H&E; (**f**) skeletal muscle with focal inflammation (arrows) (X400) H&E.

### Ratio 1:5 (two injected cohabitant fish: six non-injected cohabittee fish)

***Injected cohabitant fish***

Histopathology examination of both fish from this treatment group showed both fish were positive for *S. agalactiae* (fish 1 & 2, Appendix 9). Both fish had marked pathology consistent with infection with *S. agalactiae*, including meningitis, choroiditis, iritis, pericarditis, enteritis, gastritis, branchiitis, nephritis, serositis, peritonitis, splenic congestion (Fig. 44a, b, c, d, e & f). The presence of a large amount of melanin and haemodiserin in many major organs and tissues including the spleen, head kidney, caudal kidney and heart, also indicates the fish may have undergone haemolysis of red blood cells.

***Non-injected cohabittee fish***

Histopathology examination of all six fish from this treatment group showed four of six fish (fish 1, 2, 4 & 6) were positive for S. *agalactiae*. These fish had a very mild iritis (Fig. 45c) and a mild, deep stromal keratitis. The gills also had mild branchitis, (Fig. 45). All six fish had a generalised hypertrophy of the gill lamellae chloride cells and a few aneurisms of the gill lamellae, suggesting a water-borne irritant. One fish (fish 1) had a microsporidian-like parasite in cytoplasm of hepatocye cell obvious on Gram-Glynn stain (as already described

earlier). There was some focal skeletal muscle myositis with degeneration of some fibres (Fig. 45b). Several fish had dermatitis (Fig. 45f). Gram-Glynn-positive cocci were detected in macrophages in the brain, in the meninges. There were rare, scattered necrotic cells, and increased numbers of thrombocytes, immature red blood cells and leukocytes in the blood vessel lumens of the brain. The kidney of several fish had a mild glomerulo-nephrosis, with a mixed granulomatous inflammatory infiltrate among the haematopoietic tissue, with some rare necrotic cells (Fig. 45d). There was a mild hepatitis with perivascular inflammation (Fig. 45e), and rare foci of mononuclear inflammatory cells with adjacent degenerating hepatocytes. The spleen contained a few melano-macrophage centres, with melanin scattered throughout the spleen. The heart had a mild pericarditis of the atrium and ventricle. The heart of one fish had a single macrophage containing Gram-Glynn-positive cocci in the pericardium of the ventricle. This fish also had mild myocarditis, with a few rare focal aggregates of mononuclear inflammatory cells in the myocardium, and endocarditis, with proliferation of the atrio ventricular valve.

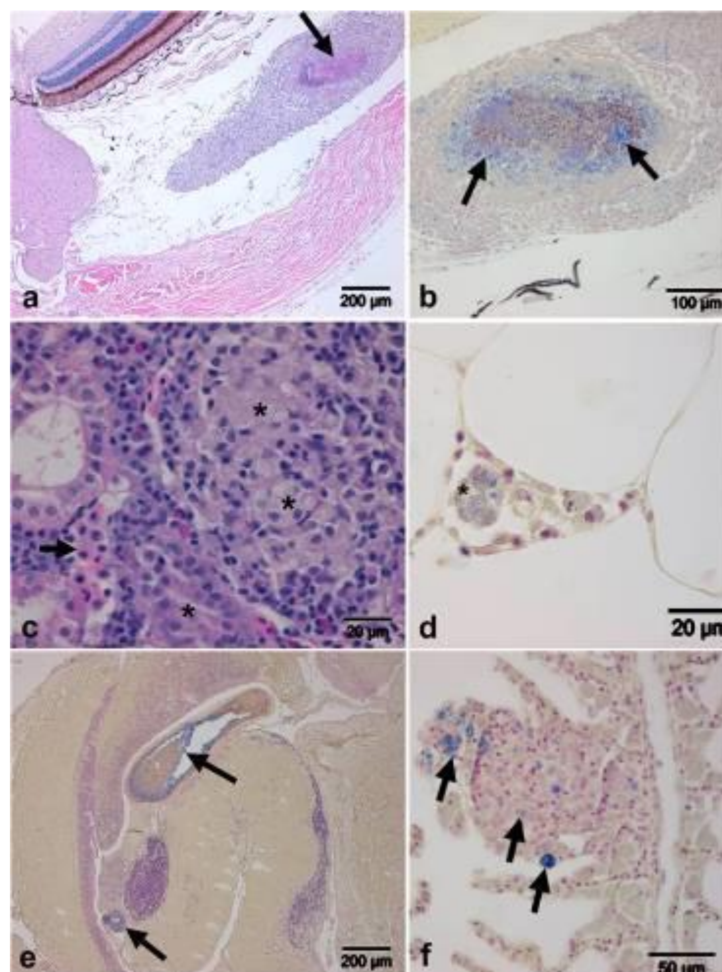


**Figure 44.** Micrographs of histological sections of the eye, caudal kidney, adipose tissue, brain and gills from juvenile grouper *E. lanceolatus*, from the cohabitation challenge trial (replicate 2, ratio 1:5, injected fish) (**a**) eye section, with large granuloma surrounded by mixed inflammatory infiltrate in choroid space (arrow) (X100) H&E; (**b**) higher power view of (a) showing Gram- Glynn positive cocci inside central core of granuloma (X200) Gram Glynn; (**c**) caudal kidney with granulomatous inflammatory lesions (*) (X1000), arrow = erythrocyte H&E; (**d**) Peritoneal adipose cells with inflammatory foci. Note large Gram-Glynn-positive cocci inside peritoneal macrophages (*) (X1000) Gram Glynn; (**e**) brain with Gram-Glynn-positive cocci (arrows) in granulomas of the meninges (arrows) (X100) Gram Glynn; (**f**) gills with Glynn-positive cocci (arrows) in macrophages in focal inflammatory lesions (arrows) (X400) Gram Glynn.

Two fish were negative for S. *agalactiae* (fish 3 & 5). Both fish had a very mild iritis, mild hepatitis, and a mild pericarditis. One fish (fish 5) had a prolific endocarditis and myocarditis with fibrosis of the myocardium.

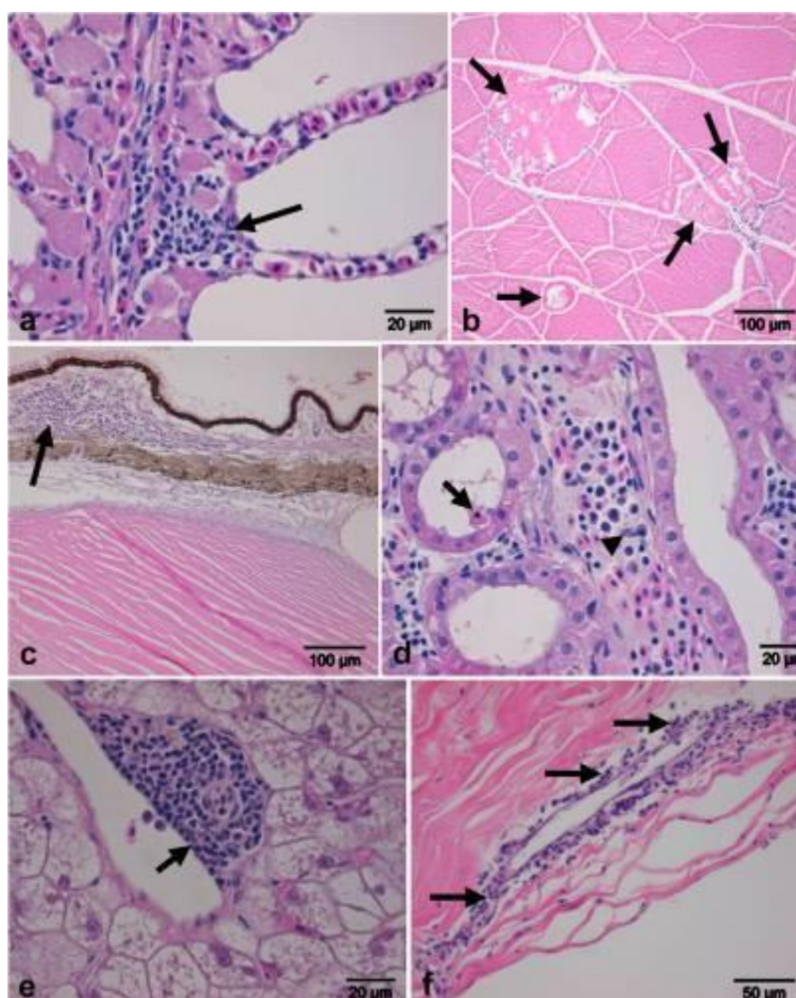


**Figure 45.** Micrographs of histological sections of the gills, skeletal muscle, eye, caudal kidney, liver and skin from juvenile grouper *E. lanceolatus*, from the cohabitation challenge trial (replicate 2, ratio 1:5, non-injected fish) (**a**) gills with focal inflammatory infiltrate (arrow) (X1000) H&E; (**b**) skeletal muscle with multiple degenerating muscle fibres (arrows) and inflammatory infiltrate (X200) H&E; (**c**) eye with mixed inflammatory infiltrate in the iris (arrow) (X100) H&E; (**d**) caudal kidney with mixed inflammatory infiltrate and necrotic cells (arrowhead) in association with Gram-Glynn-positive cocci (arrow=erythrocyte) (X1000) H&E; (**e**) liver with focal perivascular inflammatory lesion (arrow) (X1000) H&E; (**f**) skin with infiltrate of mononuclear inflammatory cells in dermal connective tissue (arrows) (X400) H&E.

### Statistical analyses and comparison of test results

In the cohabitation challenge trial, for both replicates, results for each fish using bacteriology, PCR and histology tests often differed. Individual results for each organ from each fish (and pooled test results) from PCR, bacterial isolation and histology are shown in Appendices 8 & 9. The number of fish testing positive for *S. agalactiae* by each test method (pooled results) are shown below (Tables 32 & 34). Because of logistical issues it was not always possible to obtain all three test results on every fish. Hence the total numbers vary. McNemar non-parametric tests were used to compare between tests since tests were carried out on the same subjects.

## Replicate 1

**Table. 32** Cohabitation challenge trial, replicate 1, test results for each fish (pooled organs), from PCR, histology and bacteriology.

| | | PCR | | |
|---|---|---|---|---|
| | | + | - | **Total** |
| **Histology** | + | 18 | 2 | **20** |
| | **-** | 50 | 4 | **54** |
| | **Total** | **68** | **6** | **74** |
| | | **PCR** | | |
| | | + | - | **Total** |
| **Bacteriology** | + | 0 | 1 | **1** |
| | **-** | 58 | 0 | **5** |
| | **Total** | **58** | **1** | **59** |
| | | **Histology** | | |
| | | + | - | |
| **Bacteriology** | + | 1 | 0 | **1** |
| | **-** | 19 | 40 | **59** |
| | **Total** | **20** | **40** | **60** |

For replicate 1, all three test methods were significantly different ($p<0.05$) for detecting *S. agalactiae* in internal organs of fish. PCR detected 92-98% positive fish, compared to histology which detected 27-33% and bacteriology that detected only 1.6-1.7% of positive fish (Table 33). These results indicate PCR is the most sensitive test method for detecting *S. agalactiae* from internal organs, followed by histology, then bacteriology.

**Table 33**. McNemar test results for cohabitation challenge trial, replicate 1, comparing PCR, histology and bacteriology. Replicate 1 test comparison

| **Test** | **% positive** | **% positive** |
|---|---|---|
| PCR vs histology | 91.9a* | 27.0b |
| PCR vs. bacteriology | 98.3a* | 1.6 b |
| Histology vs. bacteriology | 33.3a* | 1.7 b |

*percentages followed by a similar letter do not differ significantly ($p>0.05$)

**Table 34.** Cohabitation challenge trial, replicate 2, test results for each fish (pooled organs), from PCR, histology and bacteriology.

| | | PCR | | |
|---|---|---|---|---|
| | | + | - | **Total** |
| **Histology** | + | 15 | 3 | **18** |
| | **-** | 28 | 3 | **31** |
| | **Total** | **43** | **6** | **49** |
| | | **PCR** | | |
| | | + | - | **Total** |
| **Bacteriology** | + | 0 | 4 | **4** |
| | **-** | 25 | 2 | **27** |
| | **Total** | **25** | **6** | **31** |
| | | **Histology** | | |
| | | + | - | |
| **Bacteriology** | + | 1 | 6 | **7** |
| | **-** | 17 | 25 | **42** |
| | **Total** | **18** | **31** | **49** |

**Replicate 2.**

For replicate 2, again all three test methods were significantly different ($p<0.05$) for detecting *S. agalactiae* from internal organs of fish. PCR detected 81-88% positive fish, compared to histology which detected 37% and bacteriology which detected just 13-14% of positive fish (Table 35). These results indicate PCR is the most sensitive test method for detecting *S. agalactiae* from internal organs, followed by histology, then bacteriology.

**Table 35.** McNemar test results for cohabitation challenge trial, replicate 2, comparing PCR, histology and bacteriology.

| Replicate 2 test comparison | | |
|---|---|---|
| **Test** | **% positive** | **% positive** |
| PCR vs histology | 87.8a* | 36.7b |
| PCR vs. bacteriology | 80.6a* | 12.9b |
| Histology vs. bacteriology | 36.7a* | 14.3b |

*percentages followed by a similar letter do not differ significantly ($P > 0.05$)

## Discussion

### *Comparison of PCR and bacteriology*

In the cohabitation challenge, results for bacteriology, PCR and histology differed. Some fish that tested negative by bacteriology, tested positive by PCR, and vice versa. The results showed many fish tested negative for bacteriology, but were positive by PCR. There are several possibilities that explain the discrepancy between results from bacterial culture and PCR. There is the possibility that human error occurred when culturing bacteria from fish tissues, or during culture swabbing technique, leading to false negative results. Alternatively, there may be a low sensitivity of detection by bacteriology on blood agar for *S. agalactiae*, which is unlikely. A more likely possibility, is that PCR is detecting persistent bacterial DNA from dead or phagocytosed *S. agalactiae* in subclinical carriers, resulting in a positive PCR result. This is in agreement with the observation that most fish survived the challenge trial. Experimental observations of fish showed fish initially some fish showed signs of infection, becoming anorexic and lethargic, but after 2.5 weeks they appeared healthy, and nearly all fish survived to the end of the experiment, with no overt clinical signs of infection. This indicates these fish may be sub-clinical carriers. Furthermore it highlights that a PCR positive result alone, cannot be used to diagnose disease in fish. Histology is necessary to show pathological changes in tissues resulting from disease agents, and bacteriology results are needed to demonstrate active infection.

### *Comparison of PCR and histopathology*

In the cohabitation challenge, results for PCR and histology differed. Some fish that tested negative by histology, were positive by PCR, and vice versa. The discrepancy between results from PCR and histopathology may have resulted from human error during histological interpretation of tissue, whereby bacteria were not detected, especially if in low numbers, leading to a false negative result. Alternatively, the tissue section being read may not have any bacteria present, since histology samples only one small slice of a whole organ, leading to a false negative (test limitation). For test results where histopathology was negative and PCR was positive, the most likely explanation is that PCR was detecting dead or phagocytosed DNA of *S. agalactiae* in subclinical carrier fish. This is reflected in the experimental mortality results, whereby nearly all (except two fish) survived the experiment (Appendices 8 & 9).

***Comparison of bacteriology and histopathology***

In the cohabitation challenge, results for bacteriology and histology sometimes differed. Some fish that tested negative for *S. agalactiae* by bacteriology, but tested positive for *S. agalactiae* by histology, and vice versa. The discrepancy between results from bacteriology and histopathology may have resulted from human error occurring during either bacteriology culturing method or histological interpretation (as discussed already for oral challenge trial). It is possible bacteria were not detected, by either method, in some instances, especially if bacteria were present in very low numbers, leading to false negative results. For test results where histopathology was positive and bacteriology was negative, the most likely explanation is human error or low sensitivity of detection by bacterial culture.

***Conclusion***

PCR was consistently the most sensitive test for detecting the bacterium *S. agalactiae* from the brain, heart or spleen of fish sampled from both the oral challenge and cohabitation challenge trials. It must be noted that PCR will detect bacterial DNA, such that a positive fish may represent an infected fish, or a sub-clinical carrier of *S. agalactiae* (ie. the fish has become infected, overcome the infection, and survived). Therefore, PCR together with at least one other test method (such as histology and/or bacteriology) would be most useful for diagnosing and confirming *S. agalactiae* infection in fish.

Histopathology is a useful diagnostic tool since all organs and tissues from the fish can be examined simultaneously, and multiple disease agents can be detected. Furthermore certain pathology changes in organs and tissues are indicative of an infection process occurring. However, histopathology is time consuming and can be a subjective diagnostic test. Bacteriology is considered the gold standard for isolation of most aquatic bacterial pathogens. All diagnostic tests undertaken, however, contain the possibility of human error in methodology, leading to false positives or false negative results.

**Note:** The application of the FIHC developed at UQ would have been a useful method to further validate tests methods, and to use on histological tissue sections from all four challenge trials. However this was not possible with the Queensland Government's decision to close the DAFF Biosecurity Queensland, Tropical & Aquatic Animal Health Laboratory (TAAHL) in June 2012, during the duration of this project work. Therefore, some planned project work (test validation using FIHC on histological sections from all challenge trails) was not possible.

**Overall Conclusion**

In the oral challenge trial, there were no fish mortalities for the 5 week duration of the experiment. Two fish became moribund (from the high dose treatment group). These fish showed darkening, loss of appetite, separation from the cohort, spiral swimming, unilateral exophthalmia, ascites, and reddening of the caudal or pectoral fins (Appendix 7). In contrast to fish from the injection challenge trial (Section 6.6), fish in the oral challenge trial showed no petechial haemorrhaging on the gills, and no rectal congestion. Nearly all fish in the oral challenge trial survived the entire five weeks and had full stomachs on dissection, indicating they had been eating (Appendix 7).

In the cohabitation challenge trial, only four fish died during the five week duration of the experiment (1 fish each from Ratio 1:1 & 1:5, from the injected treatment groups, replicates 1 & 2), A further seven fish were moribund at the end of the five week experiment; six of these fish were from the injected treatment groups, and only one fish was from a non-injected

treatment group (Appendices 8 & 9). Clinical signs of infection included anorexia, spiral swimming, unilateral exophthalmia, unilateral corneal opacity, hyperaemia of the caudal or pectoral fins, empty stomach, splenomegally, a reduced size liver or spleen, and a few fish had a mouth deformity (Appendices 8 & 9). In contrast to fish from the injection challenge trial (Section 6.6), fish from cohabitation challenge trials showed no petechial haemorrhaging on the gills and no reddening of the vent. Most surviving fish had stomachs that were a third, half or full, indicating they had been eating.

Comparison of all experimental challenge trials, showed that the onset of streptococcosis for juvenile grouper differed, depending upon the route of exposure of fish to *S. agalactiae*. The onset of disease was fastest for fish injected intra-peritoneally with *S. agalactiae*, death occurring on days 2 to 3 (Section 6.6). This was similar to other transmission trials conducted on other fish species injected intra-peritoneally with *S. agalactiae* (Evans *et al*. 2002; Fiho *et al*. 2009; Geng *et al*. 2011) In comparison, the oral route of infection was much slower, with the onset of disease becoming apparent on day 26 and day 33, and by cohabitation, fish initially became moribund on day 10, but then recovered and survived the five weeks of the experiment.

The combined laboratory experimental results from the oral challenge trial including PCR, bacteriology, histopathology analysis, observed clinical signs and post mortem findings indicate that juvenile grouper *E. lanceolatus*, fed with both high and low doses of *S. agalactiae*, can become infected with *S. agalactiae* via the oral route. Similarly, the combined experimental results from the cohabitation challenge trial including PCR, bacteriology, histopathology analysis, gross clinical signs and post mortem findings indicate that healthy, non-infected juvenile grouper *E. lanceolatus*, cohabited with juvenile grouper injected with *S. agalactiae*, can become infected with *S. agalactiae* by cohabitation in the same tank, or in a different tank, within the same recirculation system (despite biological filtration, and a protein skimmer).

The laboratory test results from the oral challenge trials, showed a very low level of infection of experimental fish with *S. agalactiae*, for both replicate systems. Percentages of infected fish varied between different test methods, and between replicate systems. For replicate 1 (R1) and replicate 2 (R2) respectively; 16% and 11% of fish tested positive for *S. agalactiae* by bacterial culture; 78% (R1) and 67% (R2) of fish tested positive for *S. agalactiae* by PCR, and 33% (R1) and 22% (R2) tested positive for *S. agalactiae* by histology. These results, combined with the fact that all fish (except two) survived for the entire five week duration period of the experiment, suggests that most surviving experimental fish were infected with *S. agalactiae*, but were subclinical carriers.

Interestingly, four fish from the oral challenge trials (fish from each replicate system) had a bacterial infection with a different bacterium (a Gram-negative, rod-shaped bacterium), from histopathology examination and bacterial culture. The infection was confined to the kidneys in one fish, but was systemic, infecting multiple internal organs and tissues, in the other fish. Several Gram-negative bacteria were isolated and cultured on SBA from these fish, and were identified to species by PCR and sequencing (see results section). These were not considered significant bacterial pathogens, since all these fish were eating (had full stomachs), and showed no clinical signs of infection, (except for one fish that had red fins and was PCR positive for *S. agalactiae*, see Appendix 7).

Statistical comparison and analysis of all three laboratory test methods to detect *S. agalactiae* in fish tissues, from both the oral and cohabitation challenge trials showed differing test results. PCR was found to be the most sensitive test method for detecting *S. agalactiae* in fish.

Histopathology analysis was a most useful tool for visualising the infection process and enabled a diagnosis to be made, based on histological examination of all organs and tissues. Furthermore, bacteria could be visualised at the cellular level in multiple organs and tissues. Histopathology in Queensland grouper was consistent with that described in other fish species infected with *S. agalactiae*, from numerous other studies (Evans *et al*. 2004; Filho *et al*. 2009). In addition, some other interesting pathology was described here, which has not been previously been reported in fish infected with *S. agalactiae* in the literature.

The laboratory results, observed clinical signs and the fact that most fish survived for the five week duration period for both experiments, suggests that most surviving fish were either asymptomatic, subclinical carriers of *S. agalactiae* (showing no overt clinical signs of bacterial infection), or were only just becoming infected with the bacterium, and only just beginning to show signs of infection. Further experimental challenge trials, extending the duration of experiments, would be necessary to determine these factors.

In summary, results from both experiments indicate the transmission routes for *S. agalactiae* in juvenile Queensland grouper *E. lanceolatus,* may occur via the oral route or by cohabitation (ie. water-born).

## 6.8 Determine the presence of *S. agalactiae* in a range of marine food species that are potentially a source of infection for Queensland grouper *E. lanceolatus* in the Cairns region.

***Background***
Previous DAFF veterinary studies showed that various species of native fish in Trinity Inlet, Cairns, (where many grouper were found dead in 2008, 2009, 2010 & 2011), were infected with *S. agalactiae* (Bowater *et al*. 2012). *S. agalactiae* was detected from the heart of one wild, healthy diamond-scale mullet (*Liza vaigiensis),* two wild sick javelin grunter (*Pomadasys kaakan),* one giant sea catfish (*Arius thalassinus)* in 2009 (Bowater *et. al*. 2012). *S. agalactiae* has also been isolated from several different species of wild sick stingrays, in two disease epizootics that occurred at *Sea World* in 2009 and 2010. Some of the sick stingrays, originated from geographical regions in North Queensland (where sick and dying Queensland grouper were reported), and were translocated to *Sea World*.

Queensland grouper feed on a wide variety of prey, including fish, bait fish, mud crabs, crustaceans (prawns, crayfish), stingrays, small sharks, crocodiles and turtles, according to anecdotal information and observations made by fisheries biologists, aquarists, commercial fishermen (Lyle Squire, Cairns Marine Aquarium, *personal communication*), and necropsies conducted by DAFF Veterinarians.

This aim of this part of the project was to determine the prevalence of infection of *S. agalactiae* in a variety of wild native fish, baitfish, crustaceans, and stingrays in Trinity Inlet, Cairns, since these species are potentially a source of infection of *S. agalactiae* for wild Queensland grouper. Furthermore, we also aimed to determine whether tilapia (an introduced species) may be a source of infection of *S. agalactiae*. The Mozambique tilapia, *Oreochromis mossambicus*, is a mouth brooder that was first introduced into North Queensland over 25 years ago (Arthington *et al*. 1984) and has since spread throughout North Queensland waterways, displacing many native fish species. *O. mossambicus* was sampled from storm water drains in Cairns, adjacent to Trinity Inlet, to determine whether this species was a potential source of infection of *S. agalactiae*.

### 6.8.1 Methods
Marine species targeted in this study included native prey likely or known to be eaten by Queensland grouper (fish, prawns, mud crabs), and locally abundant in the Cairns region. One hundred species from each of five different prey types (fish, baitfish, tilapia, mud crabs, prawns) were selected for sampling (Table 36). Tilapia was chosen as a potential prey species, as this genus of fish is highly susceptible to disease outbreaks from infection with *S. agalactiae* in many countries (Chen *et al*. 2007; Evans *et al*. 2009; Hernandez *et al*. 2009; Musa *et al.* 2009; Abuseliana *et al*. 2011; Amal *et al*. 2013; Li *et al*. 2014). Detection of *S. agalactiae* in marine species was undertaken using currently available diagnostic tools (bacterial isolation and PCR).

The prevalence of *S. agalactiae* in wild fish and crustacean populations in Trinity Inlet, Cairns is currently unknown. A DAFF biometrician was consulted for sampling methodology, and an estimated conservative prevalence of 0.5 or 50% was used. The sample size required to be 80% confident that the proportion, *p*, is estimated with an error no greater than 10%, is 100 fish, of each species, based on the equation by Zar: (*n=Z^2 p q /delta^2*), ie. the fish sample size required to estimate prevalence with an error of no greater than 5%.

**Table 36. Sampling details of potential prey species for Queensland grouper in the Trinity Inlet, Cairns**

| Prey Species | No. samples | Samples | Laboratory diagnostic Tests |
|---|---|---|---|
| Mud crabs (*Scylla serrata*) | 100 | haemolymph / hindgut | Bacteriology |
| Banana prawns (*Penaeus merguiensis*) | 100 | haemolymph / hindgut | Bacteriology |
| Fish (mixed species) | 100 | brain / heart / caudal kidney / spleen | Bacteriology / PCR |
| Baitfish <30cm (mixed species) | 100 | brain / heart / caudal kidney / spleen | Bacteriology / PCR |
| Tilapia (*Oreochromis mossambicus*) | 100 | brain / heart / caudal kidney / spleen | Bacteriology / PCR |
| **TOTAL:** | **500** | | |

## Sample collection

Research assistant Ms Kerri Dyer, and senior laboratory technician, Mr Andrew Fisk were appointed to this part of the study, for two years to collect fish and crustacean samples for bacteriology and PCR analysis. Numerous fishermen of North Queensland assisted with field collection of fish, and crustaceans, and staff of Fisheries, DAFF assisted with electro-fishing in storm water drains in urban Cairns, for tilapia. Fish, baitfish, tilapia, stingray, mud crabs and prawns were collected in the Cairns region. Collection protocols undertaken by project staff were in accordance with the DEEDI (DAFF) Animal Ethics Committee Permit Reference Number CA 2010/10/472.

Samples were collected throughout the calendar year, allowing for seasonal variation in the abundance of targeted species. Fish, baitfish and one stingray were collected from various locations in Trinity Inlet, Cairns (Fig. 46). All fish and baitfish and one stingray, were euthanased using AQUI-S fish anaesthetic (as per Animal Ethics Permit Number CA2010/10/472), placed on ice and transported to TAAHL. Samples were then processed for bacteriology and Polymerase Chain Reaction (PCR).

Tilapia fish (*Oreochromis mossambicus)* were collected utilising electro-fishing back-packs operated by NFC staff. Tilapia fish were euthanased (as above) placed on ice and transported to TAAHL for bacteriology and PCR.

Mud crabs (*Scylla serrata*) were collected in baited crab pots set by professional fishers in Trinity Inlet, Cairns (Fig. 46). Crabs were cooled and sedated by refrigeration at 5$^{o}$C. Haemolymph and gut samples were collected for bacteriology. Banana prawns (*Penaeus merguiensis*) were collected in gill nets set by professional fishers in Trinity Inlet, Cairns (Fig. 46). Prawns were placed on ice and transported to TAAHL for bacteriology testing.

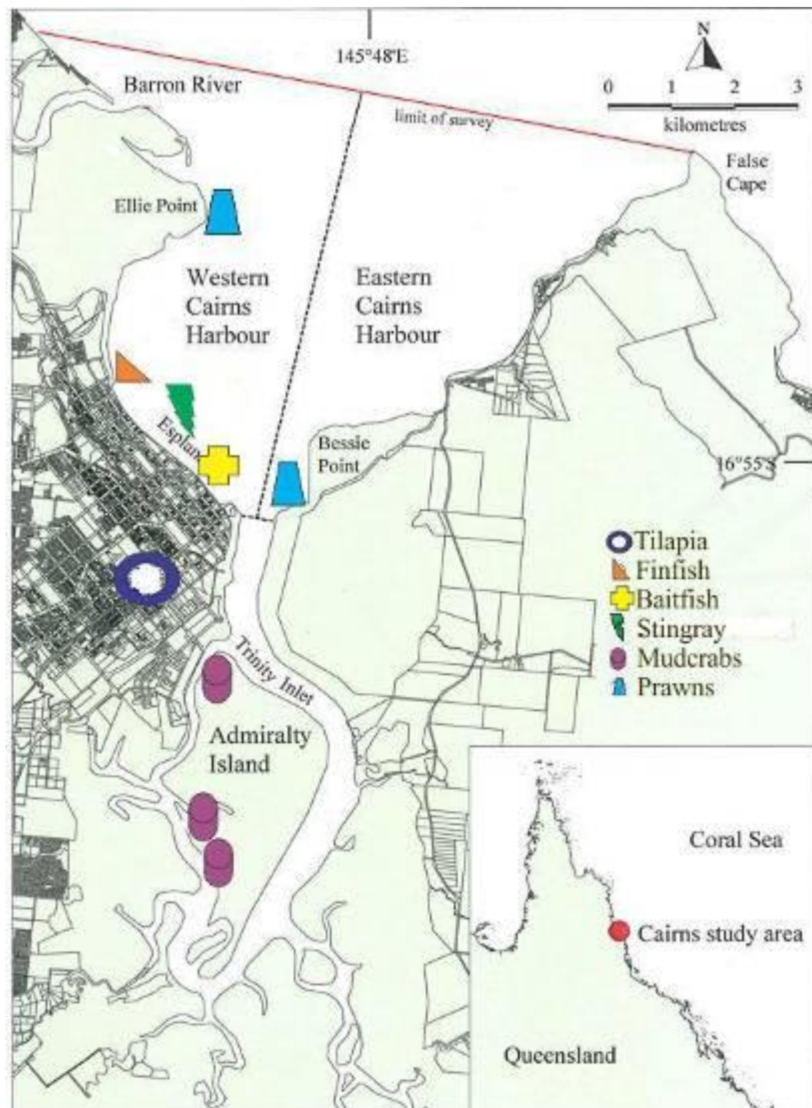


**Figure 46.** Map of Trinity Inlet, Cairns, showing sample sites used for collection of fish, baitfish, tilapia, stingray, mudcrabs and prawns from September 2010 to April 2012. The inset map shows the study site in relation to the State of Queensland, Australia.

## Necropsy and bacteriology

***(a) Fish, baitfish, tilapia & stingray***

126 fish (mixed species), 106 baitfish (mixed species), 100 tilapia (*Oreochromis mossambicus*) and one stingray (*Rhinoptera javanica*) were sampled from Trinity Inlet, Cairns, for bacteriology (Fig. 46). All fish and the elasmobranch were necropsied and sampled for bacteriology. The brain, heart, spleen and caudal kidney, were aseptically swabbed, plated directly onto sheep blood agar (SBA) enriched with bovine serum and incubated at 25$^{o}$C for up to three days. Bacterial identification of *S. agalactiae* was done using conventional biochemical tests, rapid 32 strep (API) and rapid API 20 strep Multi-test System (*BioMerieux*, France), following the manufacturer's instructions.

***(b) Mudcrabs & prawns***

A delayed wet season in the Cairns region, in 2011/2012, resulted in capture of only 84 banana prawns (*Penaeus merguiensis*) from Trinity Inlet for this study. 84 banana prawns and 105 mud crabs (*Scylla serrata*) were each sampled for bacteriology. Crabs and prawns were

sedated by chilling at 5$^{o}$C. Pleopods and heart were sterilised with 70% alcohol. A sterile 1 ml needle and syringe was used to aspirate haemolymph from a pleopod (crabs) or heart (prawns). Haemolymph was plated out directly onto SBA, and incubated at 25$^{o}$C (as outlined above). Hindgut samples were removed by gentle traction, and placed in a 3ml sterile phosphate buffered saline (PBS). Serially dilutions were made as follows: a 1:10 dilution of gut sample (0.3g) was mixed into 3ml of sterile PBS to make the first dilution ($10^{-1}$). 0.3ml of the $10^{-1}$ solution was then removed and diluted into another 3ml tube of PBS to make the second dilution ($10^{-2}$). 0.3ml of the $10^{-2}$ solution is then removed and diluted into a final tube of 3ml PBS to make the last dilution ($10^{-3}$). One drop of each of the dilutions was then streaked out onto SBA or Columbia Horse Blood Agar (HBA) with naladixic acid and colimycin (CAN), and incubated at 25$^{o}$C for 48 to 72 hrs (HBA and CNA are specific media for growth of *Streptococcu*s sp.).

**Polymerase Chain Reaction (PCR)**

PCR assay was applied to organ samples taken from 62 of 126 fish sampled, 43 of 106 bait fish sampled, 100 tilapia and one stingray. Samples of brain, heart, spleen, caudal kidney from each fish were dissected aseptically, and placed into 100% ethanol. Not all fish and baitfish were sampled for PCR, because the PCR assay was not yet developed when earlier fish samples were collected in 2010.

***Extraction of DNA from fish tissues***

DNA was extracted from fixed fish tissue as previously described in section 6.3.1.

***Optimisation of the PCR for S. agalactiae detection using AgaF/AdyR***

PCR analysis was conducted on the DNA templates using the optimised assay for fish tissue as previously outlined in section 6.3.1. A positive result was recorded if the expected amplicon size of ~845 bp was visualised.

### 6.8.2 Results

A total of 522 potential grouper food prey species (126 fish, 106 bait fish, 100 tilapia, 105 mudcrabs, 84 prawns and one stingray) were collected from Trinity Inlet, Cairns between September 2010 and April 2012 and tested for presence of *S. agalactiae* (Fig 46, Tables 37, 38, 39, 40, 41).

126 fish comprising eleven different species all tested negative for *S. agalactiae* by bacteriology and 62 fish were negative by PCR (Table 37).

**Table 37.** Bacteriology and PCR results for 126 fish sampled for *S. agalactiae* from Trinity Inlet (Cairns) from September 2010 to April 2012.

| Species (common name) | Sample number | Bacteriology (no. positive/ no. tested) | PCR (no.positive/ no. tested) |
|---|---|---|---|
| *Arius macrocephalus* (catfish) | 42 | 0/42 | 0/ 21 |
| *Neoarius graeffei* (blue catfish) | 4 | 0/4 | 0/ 0 |
| *Eleutheronema tetradactylum* (blue salmon) | 35 | 0/35 | 0/ 18 |
| *Polydactylus macrochir* (king salmon) | 21 | 0/21 | 0/ 8 |
| *Polydactylus heptadactylus* (pug nose salmon) | 6 | 0/6 | 0/4 |
| *Pomadasys opercularis* (spotted javelin fish) | 9 | 0/9 | 0/ 7 |
| *Nibea soldado* (jewfish) | 4 | 0/4 | 0/ 1 |
| *Muraenesox bagio* (pike eel) | 2 | 0/2 | 0/ 2 |
| *Scomberoides commersonnianus* (queenfish) | 1 | 0/1 | 0/ 0 |
| *Platycephalus indicus* (flathead) | 1 | 0/1 | 0/ 0 |
| *Drepane punctata* (butterfish) | 1 | 0/1 | 0/ 1 |
| **Total number:** | **126** | **0/126** | **0/62** |

### *Tilapia*

All 100 tilapia (*Oreochromis mossambicus*) samples were negative for *S. agalactiae* by bacteriology and PCR tests (Table 38).

**Table 38.** Bacteriology and PCR results for tilapia sampled for *S. agalactiae* from Trinity Inlet (Cairns) from September 2010 to April 2012.

| Species | Sample number | Bacteriology (no.positive/no. tested) | PCR (no.positive/ no. tested) |
|---|---|---|---|
| *Oreochromis mossambicus* | 100 | 0/100 | 0/100 |
| **Total number** | **100** | **0/100** | **0/100** |

### *Baitfish (< 30cm)*

All 106 baitfish tested negative for *S. agalactiae* by bacteriology and 43 fish tested negative by PCR (Table 39).

**Table 39.** Bacteriology and PCR results for baitfish species sampled for *S. agalactiae* from Trinity Inlet (Cairns) from September, 2010 to April 2012.

| Species (common name) | Sample number | Bacteriology (no.positive/ no. tested) | PCR (no.positive/ no. tested) |
|---|---|---|---|
| *Liza subviridis* (mullet) | 33 | 0/33 | 0/10 |
| *Mugil ramsayi* (mullet) | 10 | 0/10 | 0/10 |
| *Valmugil perussi* (mullet) | 5 | 0/5 | 0/0 |
| *Thryssa hamilitoni* (anchovy) | 35 | 0/35 | 0/16 |
| *Harengula macrolepis* (herring) | 15 | 0/15 | 0/6 |
| *Arrhamphus sclerolepis* (snub-nosed garfish) | 5 | 0/5 | 0/0 |
| *Saurida undosquamis* (lizardfish) | 3 | 0/3 | 0/1 |
| **Total number** | **106** | **0/106** | **0/43** |

### *Crustaceans (mudcrabs & banana prawns)*

Bacteriology analyses of 105 mudcrabs and 84 banana prawnswere negative for *S. agalactiae* (Table 40).

**Table 40.** Bacteriology results for crabs and prawns sampled for *S. agalactiae* from Trinity Inlet (Cairns) from September, 2010 to April 2012.

| Species (common name) | Sample number | Bacteriology (no.positive/ no. tested) |
|---|---|---|
| *Scylla serrata* (mudcrab) | 105 | 0/105 |
| *Penaeus merguiensis* (banana prawn) | 84 | 0/84 |

### *Elasmobranch*

Bacteriology and PCR analyses of the one stingray (*Rhinoptera javanica*) were negative for *S. agalactiae* (Table 41).

**Table 41.** Bacteriology and PCR results for 1 stingray sampled for *S. agalactiae* from Trinity Inlet (Cairns) from September, 2010 to April 2012.

| Species | Sample number | Bacteriology (no.positive/ no. tested) | PCR (no.positive/ no. tested) |
|---|---|---|---|
| *Rhinoptera javanica* (stingray) | 1 | 0/1 | 0/1 |
| **Total number** | **1** | 0 | 0 |

## Conclusion

*S. agalactiae* was not detected in any of the 522 samples of fish and crustacean collected from Trinity Inlet, Cairns, from September 2010 to April, 2012. These results indicate the prevalence of *S. agalactiae* in wild fish and crustaceans in Trinity Inlet, for the period September 2010 to April 2012 was zero. Section 6.3 showed the PCR assay has a sensitivity limit of detection of approximately 17 to 18 copies. There may be test limitations for detecting *S. agalactiae* in potential carrier fish or crustaceans e.g. if *S. agalactiae* is present in tissues of potential fish or crustaceans, beyond the level of test sensistivity, then the currently available diagnostic test method will not detect the presence of *S. agalactiae*.

Contact and liason with commercial and recreational fisherman in Cairns during sampling trips was beneficial and rewarding during the project. Public awareness of the bacterial disease in Queensland grouper was raised in fishing communities and the general public in the Cairns region, as a result of this study.

## 6.9 Determining the presence of *S. agalactiae* in food sources for Queensland grouper *E. lanceolatus* in the Cairns region.

Anecdotal information indicated Queensland grouper feed on a wide variety of prey, including fish, bait, mud crabs, prawns, crayfish, small stingrays and sharks (Lyle Squire, Cairns Marine Aquarium, *personal communication*). A small survey of twelve fish was conducted in Trinity Inlet, Cairns, in 2009, to determine whether wild fish could be a source of infection of *S. agalactiae* for Queensland grouper, by determining the presence (or absence) of *S. agalactiae*. *S. agalactiae* was detected from the heart of one healthy wild diamond-scale mullet (*Liza vaigiensis)*, stingrays from North Queensland and wild sick fish, including javelin grunter (*Pomadasys kaakan),* and giant sea catfish (*Arius thalassinus),* all previously infected with *S. agalactiae* (Bowater *et.al*. 2012).

### 6.9.1 Methods

Research assistant Ms Kerri Dyer, and senior laboratory technician, Mr Andrew Fisk were again appointed to this part of the study, for two years, to collect fish and crustacean samples for bacteriology and PCR analysis. Numerous fishermen of North Queensland assisted with collection of fish and crustaceans, and staff of Fisheries, DAFF assisted with electro-fishing in storm water drains for tilapia.

***Sampling methodology***
A senior biometrician (Angela Reid, DAFF) was consulted for sampling methodology. The prevalence of *S. agalactiae* in wild fish and crustacean populations in Trinity Inlet, Cairns is currently unknown. Therefore an estimated conservative prevalence of 0.5 or 50% was suggested. The sample size required to be 80% confident that the proportion, p, is estimated with an error no greater than 10%, is 42 fish of each species, based on the equation by Zar: (n=Z^2 p q /delta^2), ie. the fish sample size required to estimate prevalence with an error no greater than 5% (Zar, 1984).

Marine species targeted for this study were based on positive results obtained from Section 6.8. However, no positive results were obtained from crustacean and finfish sampled from Cairns, therefore, marine species targeted in this study, were based on fish species previously shown to be infected with *S. agalactiae.* This included fish of the same genus, known to be infected from a previous pilot study conducted in Cairns in 2009 (Bowater *et al*. 2012), and other fish species of this or similar fish genera, known to be susceptible to infection with *S. agalactiae* (eg. tilapia, mullet) in other countries (Evans *et al*. 2006a, 2009; Chen *et al*. 2007; Hernandez *et al*. 2009; Musa *et al.* 2009; Abuseliana *et al*. 2011; Bowater *et al*. 2012; Amal *et. al*. 2013; Huang *et al*. 2013; Li *et al*. 2014).

A proposed sampling protocol (based on the above statistical methods) of 42 samples each, of catfish, salmon, tilapia, mullet, frozen mullet and prawn species were collected (Table 42). Detection of *S. agalactiae* was undertaken using currently available diagnostic tools including bacterial isolation and PCR.

**Sample collection**
Samples were collected by DAFF technical staff, in association with commercial fishers and staff of the Northern Fisheries Centre, DAFF, Cairns. Collection protocols undertaken by project staff were in accordance with Animal Ethics Permit Number CA2010/10/472. Samples were collected throughout the calendar year to allow for seasonal variation in the

abundance of targeted species. Figure 47 shows various sampling sites within Trinity Inlet, Cairns.

**Table 42:** Sampling details of potential prey items (various fish and crustacean species) for Queensland grouper in the Cairns region.

| Prey species | no. | Tissues sampled | Laboratory test |
|---|---|---|---|
| Prawns (*Penaeus merguiensis*) | 42 | Haemolymph / gut sample | Bacteriology |
| Catfish (mixed species) | 42 | Brain, heart, caudal kidney, spleen | Bacteriology, PCR |
| Salmon (mixed species) | 42 | Brain, heart, caudal kidney, spleen | Bacteriology, PCR |
| Mullet (mixed species) | 42 | Brain, heart, caudal kidney, spleen | Bacteriology, PCR |
| Tilapia (*Oreochromis mossambicus*) | 42 | Brain, heart, caudal kidney, spleen | Bacteriology, PCR |
| Frozen baitfish (mullet species) | 42 | Brain, heart, caudal kidney, spleen | Bacteriology, PCR |
| **Total** | **252** | | |

***(a) Finfish / baitfish***
Finfish and baitfish were collected in gill nets set by professional fishermen inTrinity Inlet, Cairns (Fig. 47). All fish were euthanized using AQUI-S fish anaesthetic as outlined in Animal Ethics Permit Number CA2010/10/472, placed on ice and transported to TAAHL for bacteriology and PCR testing for *S. agalactiae.*

***(b) Tilapia***
Tilapia (*Oreochromis mossambicus)* fish were collected from urban stormwater drains and creeks in urban areas, utilising electro-fishing back-packs, operated by NFC staff. Tilapia were euthanized (as outlined above), placed on ice and transported to TAAHL for bacteriology and PCR testing for *S. agalactiae.*

***(c) Frozen baitfish***
Frozen mullet were purchased from baitfish retailers in Cairns, placed on ice and transported to TAAHL for bacteriology and PCR testing for *S. agalactiae*. The frozen mullet were sourced originally from within Trinity Inlet, Cairns, and were caught by professional fishermen.

***(d) Prawns***
Banana prawns (*Penaeus merguiensis*) were collected in gill nets set by professional fishermen based in Trinity Inlet, Cairns, placed on ice and transported to TAAHL for bacteriology testing for *S. agalactiae.*

## Necropsy and bacteriology

***(a) Finfish / mullet / frozen mullet / tilapia.***
Organs sampled from finfish, mullet, frozen mullet and tilapia for bacteriology and PCR included the brain, heart, spleen and caudal kidney. Organs were aseptically swabbed, plated directly onto sheep blood agar (SBA) and incubated at 25$^{o}$C for up to three days. Bacterial identification of *S. agalactiae* was done using conventional biochemical tests, rapid 32 strep (API) and rapid API 20 strep Multi-test System (*BioMerieux*, France), following the manufacturer's instructions.

***(b) Prawns***
Prawns were sedated by briefly chilling at 5$^{o}$C. The carapace was sterilised with 70% alcohol, and haemolymph was aspirated directly from the heart using a sterile 1 ml needle & syringe. Similarly, pleopods were sterilised with 70% alcohol and haemolymph was aspirated

using a sterile 1 ml needle & syringe. Haemolymph samples were plated directly onto SBA enriched with bovine serum and incubated at 25°C for up to three days.

Gut samples from crabs were obtained by aspirating contents from the hindgut using a sterile 5 ml syringe. Gut contents were then placed in a 3 ml sterile phosphate buffered saline (PBS) and serially diluted as follows: a 1:10 dilution of gut sample (0.3 g) was mixed into 3 ml sterile PBS to make the first dilution ($10^{-1}$). 0.3 ml of the $10^{-1}$ solution was then removed and diluted into another 3 ml PBS to make the second dilution ($10^{-2}$). 0.3 ml of the $10^{-2}$ solution is then removed and diluted in 3 ml PBS to make the last dilution ($10^{-3}$). One drop of each serial dilution was then streaked out onto a Columbia Sheep Agar (CSA) plate or Columbia Horse Blood Agar (HBA) with Naladixic acid and colimycin (CAN) plate and incubated at 25°C for 48 to 72 hrs. HBA and CNA are specific for growth of *Streptococcu*s sp. and are recommended when attempting isolation of *Streptococcus* sp. from gut samples.

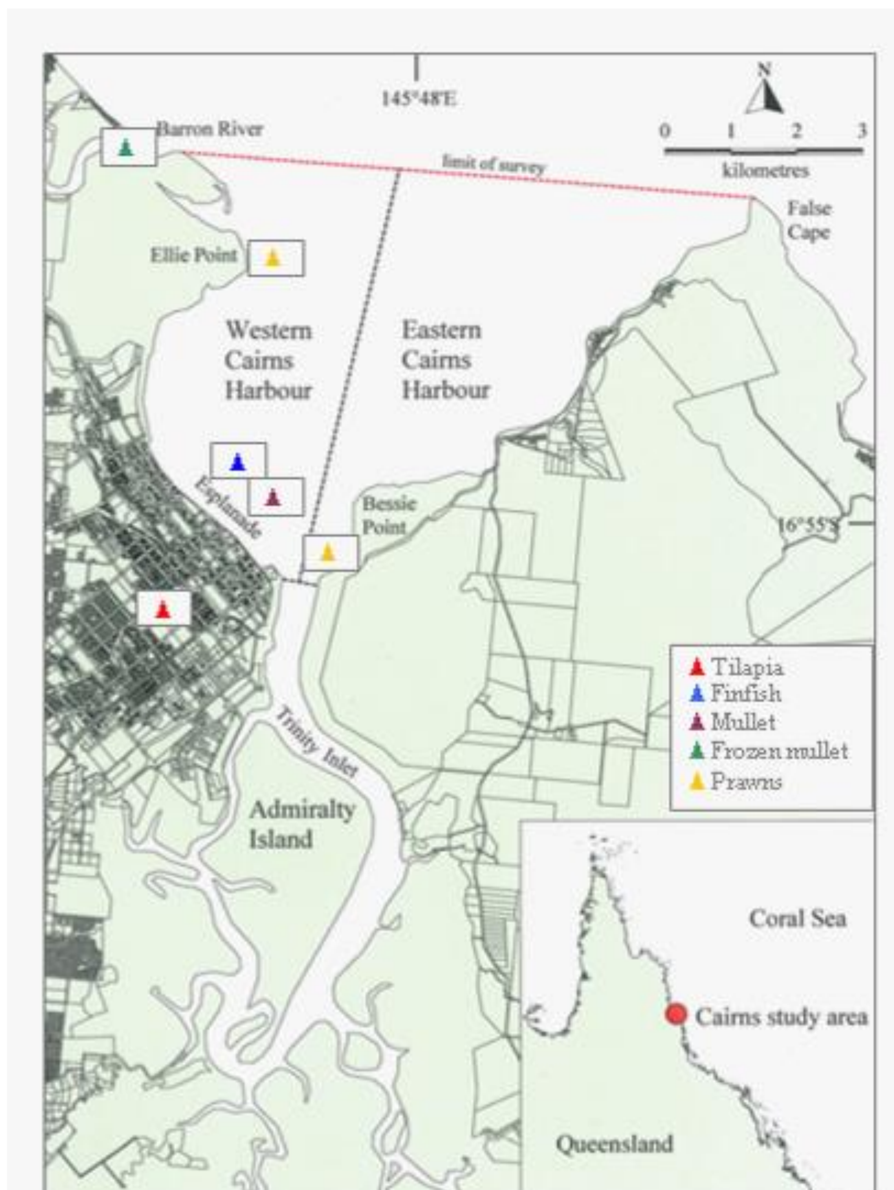


**Figure 47.** Sampling sites for collection of finfish (catfish, salmon), mullet, frozen mullet, tilapia, and prawns, within Trinity Inlet, Cairns, from September 2010 to September 2012.

**Polymerase Chain Reaction (PCR)**
Samples of brain, heart, spleen, caudal kidney were dissected aseptically from all finfish, batifish, tilapia and stingrays and placed in 100% ethanol.

**DNA extraction**
DNA was extracted from fixed fish tissue using a commercial DNA extraction kit (Roche High Pure PCR template Kit Cat 11796828001) as previously described in section 6.3.1.

**Optimisation of the PCR for *S. agalactiae* detection using AgaF/AdyR**
PCR analysis was conducted on the DNA templates using the optimised assay for fish tissue, as previously described in section 6.3.1.

### 6.9.2 Results

A total of 268 marine species (45 catfish, 45 salmon, 43 mullet, 45 frozen mullet, 45 tilapia, and 45 prawns) were collected from Trinity Inlet, Cairns between September 2010 and September 2012, sampled for *S. agalactiae* (Fig. 47; Tables 43, 44, 45, 46 and 47).

**(a) Various finfish**
All 90 samples of various fish species were negative for *S. agalactiae* using bacteriology and PCR (Table 43).

**Table 43.** Bacteriology and PCR results for sampled catfish and salmon. NT=Not tested

| Common name (species) | Sample number | Bacteriology (no. positive / no. tested) | PCR (no. positive / no. tested) |
|---|---|---|---|
| Catfish sp. *(Arius macrocephalus)* | 42 | 0 / 42 | 0 / 21 |
| Catfish sp. *(Neoarius graeffei )* | 3 | 0 / 3 | NT |
| Salmon sp. *(Eleutheronema tetradactylum)* | 25 | 0 / 25 | 0 /18 |
| Salmon sp. *(Polydactylus macrochir)* | 15 | 0 / 15 | 0 / 8 |
| Salmon sp. *(Polydactylus heptadactylus)* | 5 | 0 / 5 | 0 / 4 |
| **Total number:** | **90** | **0 / 90** | **0 / 51** |

***(b) Mullet***
All 43 mullet (various species) tested negative for *S. agalactiae* using bacteriology and PCR (Table 44).

**Table 44**. Bacteriology and PCR results for sampled mullet species. NT=Not tested

| Common name (Species) | Sample number | Bacteriology (no. positive/ no. tested) | PCR (no. positive/ no. tested) |
|---|---|---|---|
| Mullet sp. (*Liza subviridis)* | 28 | 0 / 28 | 0 / 10 |
| Mullet sp.*(Mugil ramsayi)* | 10 | 0 / 10 | 0 / 10 |

| Mullet sp. (*Valamugil perussi)* | 5 | 0 / 5 | NT |
|---|---|---|---|
| **Total number** | **43** | **0 / 43** | **0 / 20** |

***(c) Tilapia***

All tilapia were negative for *S. agalactiae* using bacteriology and PCR (Table 45).

**Table 45.** Bacteriology and PCR results for sampled tilapia.

| **Common name (Species)** | **Sample number** | **Bacteriology (no. positive/ total no. tested)** | **PCR (no. positive/ total no. tested)** |
|---|---|---|---|
| Tilapia *(O. mossambicus)* | 45 | 0 / 45 | 0 / 45 |

***(d) Frozen mullet***

All frozen bait tested negative for *S. agalactiae* using bacteriology (Table 46).

**Table 46.** Bacteriology results for sampled frozen mullet. NT=Not tested

| **Common name (species)** | **Sample number** | **Bacteriology (no. positive/ total no. tested)** | **PCR** |
|---|---|---|---|
| Mullet (*Vagamugil scheli*) | 45 | 0 / 45 | NT |

***(e) Prawns***

All prawns tested negative for *S. agalactiae* using bacteriology (Table 47).

**Table 47.** Bacteriology results for sampled prawn species. NT=Not tested

| **Common name (species)** | **Sample number** | **Bacteriology (no. positive/ total no. tested)** | **PCR** |
|---|---|---|---|
| Banana prawn *(Penaeus merguiensis)* | 45 | 0 / 45 | NT |

## Statistical conclusions

All samples returned zero fish infected with *S. agalactiae*. Thus the sample proportion (X) in all cases is zero. However, there could be some error in this figure, so 95% lower and upper confidence limits for the true proportion were calculated (using the method described in Zar, 1984). The lower confidence limits are zero. Confidence limits are expressed as a proportion or percentage (see Table 48, according to Zar, 1984).

***(a) All species combined***

For all marine species combined, X=0, n=268; thus the estimated proportion of infected fish and prawns was 0/268 = 0. The 95% confidence interval is 0, 0.0137 (or 0, 1.37%). From these results it is reasonable to conclude we are 95% confident that the true proportion of all fish and banana prawns in Trinity Inlet infected with *S. agalactiae* lies between 0 and 0.0137.

***(b) Individual species***

For each fish category, 45 prawns (*Penaeus merguiensis)*, 45 catfish (three different species), 45 salmon (three different species), 45 mullet (three different species), 45 tilapia *(O. mossambicus)* and 45 frozen bait mullet (*Vagamugil scheli*) sampled in Trinity Inlet; X =0, n = 45; *P* = 0/45 = 0; the 95% confidence interval for *P* is 0, 0.0787 (or 0, 7.87%) for each of these species. From these results it is reasonable to conclude we are 95% confident that the true proportion of prawns, catfish, salmon, mullet, tilapia and frozen bait mullet infected with *S. agalactiae* in Trinity Inlet lies between 0 and 0.0787 (Table 48).

For the 43 mullet sampled in Trinity inlet, X = 0, n=43; *P* = 0/43 = 0; the 95% confidence interval for *P* is 0, 0.0822 (or 0, 8.22%) for this species. From these results it is reasonable to conclude we are 95% confident that the true proportion of mullet infected with *S. agalactiae* in Trinity Inlet lies between 0 and 0.0822 (Table 48).

**Table 48.** Upper confidence limits (based on the equation by Zar, 1984) for individual prawn and fish species, sampled in Trinity Inlet, from September, 2010 to September 2012.
X= sample proportion; n = number sampled; v1=2; v2=nx2

| Common name (Species) | X | n | v1 | v2 | F | 95% Upper CI as ppn | 95% Upper CI as % |
|---|---|---|---|---|---|---|---|
| **Banana prawn (*P. merguiensis*)** | 0 | 45 | 2 | 90 | 3.844 | 0.0787 | 7.87 |
| **Catfish (2 species)** | 0 | 45 | 2 | 90 | 3.844 | 0.0787 | 7.87 |
| **Salmon (3 speceis)** | 0 | 45 | 2 | 90 | 3.844 | 0.0787 | 7.87 |
| **Mullet (3 species)** | 0 | 43 | 2 | 86 | 3.852 | 0.0822 | 8.22 |
| **Tilapia (*O. mossambicus*)** | 0 | 45 | 2 | 90 | 3.844 | 0.0787 | 7.87 |
| **Frozen bait, mullet (*V. scheli*)** | 0 | 45 | 2 | 90 | 3.844 | 0.0787 | 7.87 |

## Conclusion

*S. agalactiae* was not detected in 268 marine animals comprising; 45 catfish (two different species), 45 salmon (three different species), 45 tilapia, 43 mullet (three different species), 45 frozen mullet (*V. scheli*), or 45 banana prawns (*Penaues merguiensis*), sampled from Trinity Inlet, Cairns, from September 2010 to September 2012, using current diagnostic methodology available (bacteriology and PCR) for detecting S. *agalactiae.*

Public awareness of the bacterial disease in Queensland grouper was raised as a result of sampling numerous coastal study sites used during the course of this study. This occurred through contact and liason with recreational and commercial fishermen, indigenous communities, the general public, the tourism industry and the seafood industry, at numerous coastal regions along the east coast of Queensland, and in the Gulf of Carpentaria over the course of this study.

## 6.10 Determine the distribution and prevalence of *S. agalactiae* in fish along the North Queensland coast

### 6.10.1 Methods

Research assistant Ms Kerri Dyer, and Senior Laboratory Technician, Mr Andrew Fisk were again appointed to this part of the study, for two years to collect fish and crustacean samples for bacteriology and PCR analysis. Numerous commercial fishermen of North Queensland assisted with field collection of fish, and crustaceans and staff of Fisheries, DAFF also assisted with some field sampling.

**Sampling methodology**

***(a) Targeted surveillance for S. agalactiae***

Marine species selected for targeted surveillance to detect *S. agalactiae*, were to be based on positive species results obtained from Section 6.9**.** However, no positive results were obtained from crustacean or finfish samples from the surveillance studies undertaken.

Due to WPH&S issues it was not a realistic proposition to capture and sample 45 stingrays. 45 tilapia were collected as a replacement species based on the susceptibility of this genus of fish to infection with *S. agalactiae* (Evans *et al*. 2006a, 2009; Chen *et al*. 2007; Hernandez *et al*. 2009; Musa *et al.* 2009; Abuseliana *et al*. 2011; Bowater *et al*. 2012; Amal *et. al*. 2013; Huang *et al*. 2013; Li *et al*. 2014).

A pilot study done by DAFF in Trinity Inlet in 2009 (Bowater *et al*. 2012) showed a healthy mullet tested positive for *S. agalactiae*. Based on this finding, mullet was selected as a sample species, for this study.

For targeted surveillance, a sample size of 42 mullet was selected (see sections 6.8, 6.9 for statistical sampling methods), from each of four different geographical locations along the North Queensland Coast; Weipa, Cairns, Townsville, and Proserpine (Table 49, Figure 48). Another baitfish species was to be selected, only if mullet were unavailable due to seasonal variation in abundance.

Detection of *S. agalactiae* in all marine species was undertaken using bacterial isolation and PCR (as previously outlined in Section 6.9).

**Table 49.** Targeted surveillance sampling details for mullet species from Weipa, Cairns, Townsville, and Proserpine.

| Species | No. samples | Tissues sampled | Laboratory diagnostic Tests done |
|---|---|---|---|
| Mullet (mixed species) | 42 | brain/heart/caudal kidney/spleen | Bacteriology/PCR |
| **Total** | **168** | | |

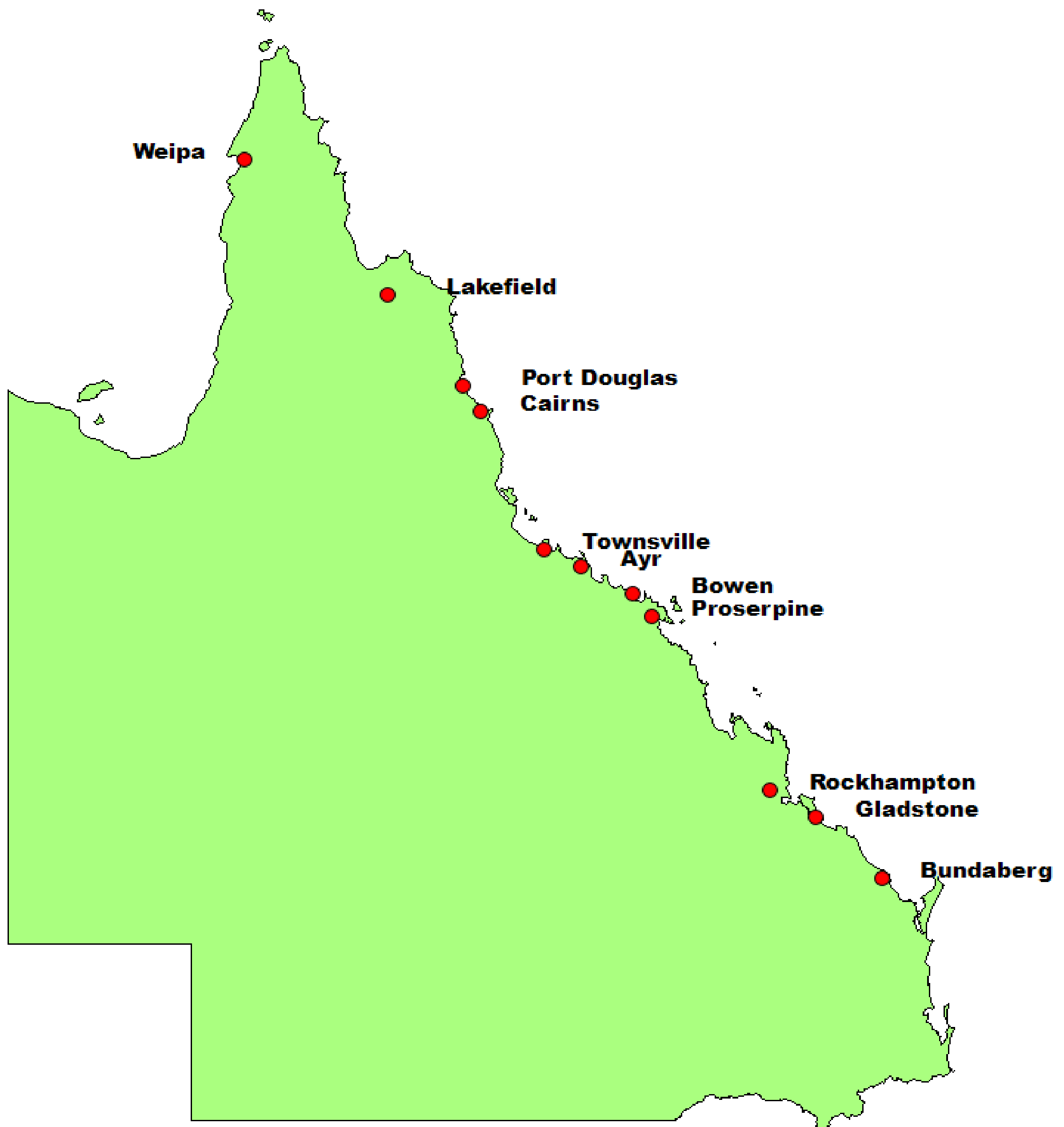


**Figure 48.** Map of Queensland showing sample collection sites, to determine the distribution and prevalence of *S. agalactiae* in fish and crustacean populations along the Queensland coast from September 2010 to December 2012.

***(b) Extra fish samples***

A total of 363 other samples comprising; 24 finfish, 6 tilapia, 154 mullet (69 were frozen mullet obtained from a bait shop) and 179 mudcrabs, were obtained variously, from Weipa, the Normanby River (Lakefield National Park), Tongue Reef (GBR, Port Douglas), Ayr, Bowen, Proserpine, Rockhampton, Gladstone and Bundaberg (Figure 48). These samples were collected for other diagnostic reasons (as outlined below), but were also tested *ad hoc* for *S. agalactiae.* These extra samples allowed expansion of the geographical study area for detection of *S. agalactiae* and an increased species diversity study.

## Sample collection

### Targeted surveillance for *S. agalactiae*

Baitfish (mullet) was collected in gill nets set by professional fishers from Weipa, Cairns, Townsville, and Proserpine (Fig. 48). All fish were euthanized using AQUI-S fish anaesthetic as outlined in Animal Ethics Permit Number CA2010/10/472 then placed on ice and transported to TAAHL for bacteriology and PCR testing for *S. agalactiae* (as outlined previously). PCR testing was not undertaken on all organ samples, when correct preservation procedures for PCR were not possible for some fish.

### Extra fish samples (finfish, baitfish, frozen baitfish, mudcrabs)

A range of different species of finfish, tilapia, baitfish, frozen baitfish and mudcrabs were also sent to TAAHL by professional or recreational fishers and Regional Biosecurity staff for other diagnostic purposes (as outlined below). These samples were submitted from Weipa, the Normanby River (Lakefield National Park), Tongue Reef (GBR, Port Douglas), and coastal areas of Ayr, Bowen, Proserpine, Rockhampton, Gladstone and Bundaberg (Figure 48). These samples were also tested for *S. agalactiae* utilising bacteriology or PCR analysis.

***Weipa & the Gulf of Carpentaria***

A small fish kill occurred near Pormpuraaw, the Gulf of Carpentaria in 2011 involving mullet. Eight frozen mullet (*Liza sp*.) with red skin lesions were submitted for diagnostic purposes, by gross and histopathology (with a diagnosis of Epizootic Ulcerative Syndrome confirmed). Fish were defrosted, and samples taken from the brain, eye, heart, kidney and spleen for bacteriology and PCR testing for *S. agalactiae.* A variety of other wild marine fish, including; five mangrove jack *(Lutjanus argentimaculatus),* ten large-scale catfish (*Arius macrocephalus*), ten brown-spotted grouper (*Epinephalus tauvina*), and 22 mullet (unidentified *Liza sp*.) were caught by hook and line by commercial fishermen from Albatross Bay, Weipa. Frozen heads from these fish were collected and sent on ice to TAAHL for testing. Frozen heads were defrosted and brains were dissected out and tested for *S. agalactiae* using PCR and bacteriology.

***Normanby River, Lakefield National Park***

A large-scale fish kill occurred on 15-16 July 2011, in a wide part of the Normanby River, upstream from Lakefield National Park. This fish kill involved multiple fish species including barramundi (*Lates calcarifer*), catfish and eel-tail catfish (*Neosilurus* sp.). Water temperatures were $22^0$C at the time of sampling, and no human activity other than extensive beef cattle grazing reported in the area at the time of sampling (refer to http://www.fishingworld.com.au/news/fish-kill-attributed-to-white-spot-disease). Two barramundi (one frozen, another freshly dead on ice), were submitted to TAAHL for diagnostic purposes (histology, bacteriology). Both fish had reddened skin lesions at the base of the pectoral fins, ventral and caudal fins. Gill and skin smears showed a few skin flukes & numerous whitespot (*Ichthyophthirius multifiliis*) trophonts on the gills. Two frozen eel-tail catfish (*Neosilurus* sp) were also submitted for disease testing. The catfish was reported bleeding from the skin when removed from the water and had widespread petechial & ecchymotic haemorrhages on the skin, gills, body, and eyes, indicating possible bacterial involvement (Fig. 49). Both fish had gross signs of whitespot infestation on the gills and skin, with skin and gills scrapings showing large numbers of *Ichthyophthirius multifiliis* trophonts. All four fish were tested by bacteriology and histology for diagnostic purposes.

Internal organs and tissues were sampled from all four fish and tested for *S. agalactiae* by bacteriology and PCR (as previously outlined in section 6.9).

**Figure 49.** Eeltail catfish from a fish kill in Lakefield National Park, July 2011, with extensive ecchymotic and petechial haemorrhaging of the skin from bacterial septicaemia (*Aeromonas sobria*). This fish also had an infestation with whitespot (*Ichthyophthirius multifiliis*).

***Tongue Reef, Great Barrier Reef, Port Douglas.***

A freshly dead hump-headed maori wrasse (*Cheilinus undulatus*) (3350 g, 930 mm) was collected by Queensland Boating & Fishing Patrol Officers from illegal fishing activity on Tongue Reef (GBR, Port Douglas) on 24 March, 2011 (Fig. 50). The fish was frozen and submitted on ice to TAAHL for testing for *S. agalactiae* by bacteriology, histology & PCR. A full set of organs and tissues were collected including the eyes, brain, liver, kidney, spleen and heart.

***Rockhampton, Gladstone, Bundaberg***

Fish collected from Bundaberg, Rockhampton & Gladstone were part of the Gladstone Fish Kill Investigation, and were collected by commercial fishermen or DAFF Fisheries Officers. Fish were sampled on site, and the brains only were removed and preserved in ethanol for testing for *S. agalactiae* using PCR. Other organs and tissues were also collected from these fish by DAFF Officers for histopathology analysis but results are not included in this study.

**Figure 50.** Hump-headed Maori Wrasse (*Cheilinus undulatus*) from Tongue Reef, Great Barrier Reef, 2011, sent to TAAHL for post mortem and testing for *S. agalactiae.*

### 6.10.2 Results

**Targeted surveillance for *S. agalactiae***

A total of 179 baitfish (159 mullet and 20 whiting) were collected along the Queensland coast as part of targeted surveillance for *S. agalactiae* from Weipa in the Gulf of Carpentaria to as far south as Proserpine on the east coast, between September 2010 and September 2012. All 159 mullet (45 from Weipa, 43 from Cairns, 45 from Townsville and 46 from Proserpine tested negative for *S. agalactiae* using PCR and bacteriology (Tables 50, 51, 52 & 53).

***Weipa (Gulf of Carpentaria)***
The 45 mullet sampled as part of the targeted surveillance for *S. agalactiae*, all tested negative for *S. agalactiae* by PCR and bacteriology (Table 50). Extra samples including 49 marine fish (comprising 5 different fish species) and 11 mudcrabs (comprising two different species) were collected from Weipa for routine disease diagnostic purposes. All fish and crustaceans tested were negative for *S. agalactiae*, using bacteriology or PCR (Table 50).

**Table 50.** Bacteriology and PCR results for mullet*, other finfish, frozen baitfish and mudcrabs sampled for *S. agalactiae* from Weipa from September 2010 to September 2012.
*targeted surveillance species collected, NT=Not tested, #brain only tested by PCR & bacteriology

| Prey Species | No. of samples | Bacteriology (no. pos / no. tested) | PCR (no. pos / no. tested) |
|---|---|---|---|
| Large-Scale Catfish # *Arius macrocephalus* | 10 | 0 / 10 | 0 / 10 |
| Mangrove Jack # *Lutjanus argentimaculatus* | 5 | 0 / 5 | 0 / 5 |
| Brown-spotted grouper # *Epinephalus tauvina* | 4 | 0 / 4 | 0 / 4 |
| Mullet *Liza sp* | 8 | 0 / 8 | 0 / 8 |
| Mullet * *Liza* sp. | **45** | 0 / 45 | 0 / 45 |
| Mullet # *Liza* sp. | 22 | 0 / 22 | 0 / 22 |
| Mud crab sp. *Scylla Alivacia* | 4 | 0 / 4 | NT |
| Mud crab sp. *Scylla serrata* | **7** | 0 / 7 | NT |
| **Total** | **105** | **0/105** | **0/ 94** |

### *Cairns*

Forty three mullet sampled (part of targeted surveillance), from Trinity Inlet, Cairns, tested negative for *S. agalactiae* using bacteriology and PCR analyses (Table 51).

**Table 51.** Bacteriology and PCR results for mullet species sampled for *S.agalactiae* from Trinity Inlet (Cairns) from September, 2010 to September 2012.
*targeted surveillance species collected. * targeted surveillance species collected, NT=Not tested

| Species (common name) | Sample number | Bacteriology (no. pos / no. tested) | PCR (no. pos / no. tested) |
|---|---|---|---|
| Mullet * *Liza subviridis* | 28 | 0 / 28 | 0 / 10 |
| Mullet * *Mugil ramsayi* | 10 | 0 / 10 | 0 / 10 |
| Mullet * *Valamugil perussi* | 5 | 0 / 5 | NT |
| **Total number** | **43** | **0 / 43** | **0 / 20** |

### *Townsville*

Forty five mullet (part of targeted surveillance), sampled from Townsville, tested negative for *S. agalactiae*, using bacteriology or PCR (Table 52). A further 25 fish and 14 mud crabs also tested negative for *S. agalactiae* using bacteriology or PCR (Table 52).

**Table 52.** Bacteriology and PCR results for tilapia, mullet, frozen mullet and mudcrabs sampled for *S. agalactiae* from Townsville (coastal regions) from September 2010 to September 2012**.**
*targeted surveillance species collected, NT=Not tested

| Prey Species | No. of samples | Bacteriology (no. pos / no. tested) | PCR (no. pos / no. tested) |
|---|---|---|---|
| Mullet * *Liza subviridis* | **45** | 0 / 45 | 0 / 45 |
| Mullet (frozen) *Mugil cephalus* | 19 | 0 / 19 | NT |
| Tilapia *Oreochromis mossambicus* | 6 | 0 / 6 | NT |
| Mud crab *Scylla serrata* | 14 | 0 / 14 | NT |
| **Total** | **84** | **0 / 84** | **0 / 45** |

### *Proserpine*

Forty six bait fish (mullet and whiting) tested (part of targeted surveillance), sampled from Proserpine, tested negative for *S. agalactiae* using bacteriology and PCR analyses (Table 53). Six mudcrabs, collected for diagnostic purposes, also tested negative for *S. agalactiae* from bacteriology (Table 53).

**Table 53.** Bacteriology and PCR results for baitfish and mudcrabs sampled for *S. agalactiae* from Proserpine (coastal region), from September 2010 to September 2012.
* targeted surveillance species collected, NT=Not tested

| Prey Species | No. of samples | Bacteriology (no. pos / no. tested) | PCR (no. pos / no. tested) |
|---|---|---|---|
| Mullet * *Liza subviridus* | **26** | 0 / 26 | 0 / 26 |
| Whiting * *Silligo cillata* | **20** | 0 / 20 | 0 / 20 |
| Mud crab sp. *Scylla serrata* | 6 | 0 / 6 | NT |
| **Total** | **52** | **0 / 52** | **0 / 46** |

## Exta fish samples (finfish, baitfish, mudcrabs)

A further 363 marine animals were tested for *S.agalactiae* including; 10 freshwater or brackish water fish species (2 barramundi, 6 tilapia , 2 catfish), 174 marine fish (various species) and 179 mud crabs. Samples were obtained from various geographical regions, both in the Gulf of Carpentaria and on the east coast of northern Queensland, and were sampled and tested according to methods previously outlined in Section 6.10.

#### *Normanby River, Lakefield National Park*

Three of the four fish (two barramundi and one eeltail catfish) sampled from the Normanby river fish kill, sampled for routine laboratory diagnostic testing, tested negative for *S. agalactiae* by PCR and bacteriology. One eeltail catfish fish tested positive for *S. agalactiae* using PCR (Table 54). An amplicon was obtained from the brain sample. This PCR result was not confirmed by bacterial culture, which was negative for *S. agalactiae*. The genetic sequence of the amplicon could not be determined with sequencing.

**Table 54.** Bacteriology and PCR results for finfish sampled for *S. agalactiae* from a fish kill in the Normanby River, Lakefield National Park, July 15th and 16th, 2012.

| Prey Species | No. of samples | Bacteriology (no. pos / no. tested) | PCR (no. pos / no. tested) |
|---|---|---|---|
| Barramundi *Lates calcarifer* | 2 | 0 / 2 | 0 /2 |
| Eeltail Catfish *Neosilurus sp* | 2 | 0 / 2 | 1/ 2 |
| **Total** | **4** | **0 / 4** | **1/ 4** |

### *Port Douglas*

A single hump-headed maori wrasse (*Cheilinus undulatus*) from the Great Barrier Reef, Port Douglas tested negative for *S. agalactiae* using bacteriology and PCR analyses (Table 55).

**Table 55.** Bacteriology and PCR results for fish sampled for *S. agalactiae,* from Tongue Reef, near Port Douglas from September 2010 to September 2012.

| Prey Species | No. of samples | Bacteriology (no. pos / no. tested) | PCR (no. pos / no. tested) |
|---|---|---|---|
| Hump-headed Maori Wrasse *Cheilinus undulatus* | 1 | 0 / 1 | 0 / 1 |

### *Ayr*

Thirty mud crabs (*Scylla serrata*) from Ayr were negative for *S. agalactiae* using bacteriology (Table 56).

**Table 56.** Bacteriology and PCR results for mudcrabs, sampled for *S. agalactiae* from Ayr from September 2010 to September 2012. NT=Not tested

| Prey Species : | No. of samples | Bacteriology (no. pos / no. tested) | PCR |
|---|---|---|---|
| Mud crab *Scylla serrata* | 30 | 0 / 30 | NT |

### *Bowen*

Fifty frozen mullet baitfish from Bowen were negative for *S.agalactiae* using bacteriology and PCR analyses (Table 57).

**Table 57.** Bacteriology and PCR results for frozen baitfish sampled for *S. agalactiae* sampled in Bowen from September 2010 to September 2012.

| Prey Species | No. of samples | Bacteriology (no. pos / total no. tested) | PCR (no. pos / total no. tested) |
|---|---|---|---|
| Mullet (frozen) *Valamugil cunnesius* | 50 | 0 / 50 | 0 / 50 |

### *Rockhampton*

Ten mullet and nine mudcrabs sampled from Rockhampton tested negative for *S. agalactiae* using bacteriology or PCR analyses (Table 58).

**Table 58.** Bacteriology and PCR results for mullet (baitfish) and mudcrabs, sampled for *S.agalactiae* in Rockhampton from September 2010 to September 2012. NT=Not tested #brain only tested

| Prey Species | No. of samples | Bacteriology (no. pos / no. tested) | PCR (no. pos / no. tested) |
|---|---|---|---|
| Mullet # *Mugil cephalus* | 10 | NT | 0 / 10 |

| | | | |
|---|---|---|---|
| Mud crab *Scylla serrata* | 9 | 0 / 9 | NT |
| **Total** | **19** | **0 / 9** | **0 / 10** |

### *Gladstone*
Thirty five mullet and 99 mud crabs from Gladstone tested negative for *S. agalactiae* using PCR or bacteriology (Table 59). One mullet* (*Mugil cephalus*) had a suspect positive (very faint positive amplicon), observed from the brain when tested with PCR. Bacteriology was not done on this sample. The genetic sequence of the amplicon could not be determined with sequencing. It is presently unknown if this result represent a significant finding or was a result of non-specific reactors to the sample.

**Table 59.** Bacteriology and PCR results for baitfish and mudcrabs, sampled for *S. agalactiae* in Gladstone from September 2010 to September 2012. NT=Not tested #brain only tested

| Prey Species | No. of samples | Bacteriology (no. pos / no. tested) | PCR (no. pos / no. tested) |
|---|---|---|---|
| Mullet # **Mugil cephalus* | 35 | NT | 1 / 35 |
| Mud crab *Scylla serrata* | 99 | 0 / 99 | NT |
| **Total** | **134** | **0 / 99** | **1 / 35** |

### *Bundaberg*
Ten mullet and ten mud crabs from Bundaberg tested negative for *S. agalactiae* using bacteriology and PCR analyses (Table 60).

**Table 60.** Bacteriology and PCR results for baitfish and mudcrabs, sampled for *S. agalactiae* in Bundaberg from September 2010 to September 2012. NT=Not tested, #brain only tested.

| Prey Species | No. of samples | Bacteriology (no. pos / no. tested) | PCR (no. pos / no. tested) |
|---|---|---|---|
| Mullet # *Mugil cephalus* | 10 | NT | 0 / 10 |
| Mud crab *Scylla serrata* | 10 | 0 / 10 | NT |
| **Total** | **20** | **0 / 10** | **0 / 10** |

## Conclusion

### Targeted surveillance
*S. agalactiae* was not detected, using current diagnostic methodology (Bacteriology and PCR) from targeted surveillance of 179 wild, healthy mullet (the target species), from four different geographical regions along the Queensland coast, Weipa (in the Gulf of Carpentaria), Cairns, Townsville and Proserpine (on the east coast).

For targeted surveillance, to detect *S. agalactiae* in fish in North Queensland, (using mullet as a target species), X=0, and n=179, the estimated proportion of diseased fish was 0/179 = 0. The 95% confidence interval was 0, 0.0204 (or 0, 2.04%). From these results it is reasonable to conclude that we are 95% confident that the true proportion of fish infected with *S. agalactiae* in mullet along the North Queensland coast lies between 0 and 0.0204 (Table 61). *S. agalactiae* is therefore not highly prevalent in wild healthy mullet populations (using currently available diagnostic tools) along the North-Eastern coast of Queensland and Gulf of Carpentaria.

**Table 61.** Calculation of upper confidence limits, based on the equation by Zar (1984), for mullet sampled from Cairns only (Section 6.8), North Queensland coastal regions (Section 6.9: targeted surveillance), mullet from Bowen, and mudcrabs from Gladstone Harbour (section 6.10: extra samples) from September, 2010 to September 2012.
X=sample proportion; n=number sampled; v1=2;v2=nx2 ; NQ=North Queensland.

| Coastal Region of Queensland (fish type) | X | n | v1 | v2 | F | 95% Upper CI as ppn | 95% Upper CI as % |
|---|---|---|---|---|---|---|---|
| **Trinity Inlet, Cairns (Objective 2)** | 0 | 268 | 2 | 536 | 3.714 | 0.0137 | 1.37 |
| **NQ (mullet) (Objective 3a)** | 0 | 179 | 2 | 358 | 3.727 | 0.0204 | 2.04 |
| **Bowen (mullet) (Objective 3b)** | 0 | 50 | 2 | 100 | 3.828 | 0.0711 | 7.11 |
| **Gladstone (mudcrabs) (Objective 3b)** | 0 | 99 | 2 | 198 | 3.758 | 0.0366 | 3.66 |

## Extra samples tested

*S. agalactiae* was not detected from the extra 363 wild marine species tested (174 marine fish and 179 crustaceans) and 10 freshwater/brackish water fish (barramundi, tilapia, eeltail catfish), using specific *S. agalactiae* bacteriological and PCR methods. These samples spanned a coastal area covering over 3300 km, and came from as far south as Bundaberg, and from regions where large fish kills had occurred (Weipa, Normanby River, Gladstone, Rockhampton) and from regions along coastal Queensland where Queensland grouper had previously been reported dead including; Bundaberg, Gladstone, Rockhampton, Proserpine, Bowen, Ayr, Townsville, Cairns and Port Douglas and Weipa (Bowater *et al*. 2012).

In this study, extra samples obtained for a particular species, including crabs from Gladstone and mullet from Bowen, met or exceeded the required number (n = 42) to estimate prevalence (see section 6.8). Therefore statistical analysis was done on these.

### *Bowen (mullet)*

For frozen mullet (*Valamugil cunnesius)* sampled in Bowen, X = 0, and n = 50, thus the estimated proportion of diseased fish was 0/50 = 0. The 95% confidence interval is 0, 0.0711 (or 0, 7.11%). From these results it is reasonable to conclude we are 95% confident that the true proportion of frozen mullet *V. cunnesius* infected with *S. agalactiae* in Bowen lies between 0 and 0.0711 (Table 61).

### *Gladstone (mudcrabs)*

For mudcrabs (*Scylla serrata*) sampled from the Gladstone Harbour region, X = 0, and n = 99, thus the estimated proportion of diseased fish was 0/99 = 0. The 95% confidence interval is 0, 0.0366 (or 0, 3.66%). From these results it is reasonable to conclude we are 95% confident that the true proportion of mudcrabs *S. serrata* from Gladstone harbour region, infected with *S. agalactiae* lies between 0 and 0.0366 (Table 61).

***Positive samples***
Two fish tested positive by PCR for *S. agalactiae*; an eeltail catfish *(Neosilurus* sp.) from an inland freshwater Normanby River, from a large-scale fish kill involving multiple fish species, in Lakefield National Park; and a marine mullet (*Mugil cephalus*) sampled from Gladstone.

Both fish showed a weak positive amplicon from brain tissue sampled by PCR. Bacteriology results from the brain of the catfish yielded a *Streptococcus* sp. but this species was not identified further as *S. agalactiae*. The eeltail catfish was diagnosed with a bacterial septicaemia from infection with the bacterium *Aeromonas sobria* (pure growth of *A. sobria* grown in culture from internal organs) and was also concurrently infected with a heavy infestation of whitespot disease. These diagnoses were made from both histopathology and bacteriology testing done on the four fish sampled from Lakefield National Park.

It is presently unknown if this PCR result represents a significant finding or was a result of non-specific reactors to the sample, since post mortem degradation of tissues was present in the eeltail catfish at the time of fish sampling, due to the remoteness of the location and inability to obtain freshly dead material for PCR and bacteriology. The fish was also frozen prior to transport and sampling for bacteriology and PCR testing.

In the case of the mullet sampled from Gladstone, however, the brain was sampled directly from a freshly caught wild fish and was preserved specifically into ethanol for PCR analysis.

The spurious PCR results from both fish, suggests a possibility that either mullet and/or eeltail catfish could be asymptomatic carriers of *S. agalactiae*. This was an interesting result, given that a previous pilot study conducted in Trinity Inlet in 2009 showed a healthy diamond-scale mullet (*Liza vaigiensis*), tested positive for *S. agalactiae* by bacteriology (Bowater *et al*. 2012).

These results warrant further scientific diagnostic investigation, including further targeted surveillance sampling these particular fish species, namely mullet (*Mugil cephalus*) and eeltail catfish (*Neosilurus* sp.). This may further aid in determining the distribution and prevalence of *S. agalactiae* within certain fish populations along the Queensland coast.

Further improvement of currently available diagnostic tests to detect *S. agalactiae*, including heightened test sensitivity and specificity would be useful, since test specificity and sensitivity of the currently available diagnostic tools to detect *S. agalactiae* in fish are unknown, although results from this project show PCR is the most sensitive test. Further targeted surveillance of these fish species and related fish species, using a diagnostic test with a known and possibly greater test sensitivity and specificity may identify particular fish species that are sub-clinical carriers of *S. agalactiae*, thus identifying high-risk fish species.

In Australia many large commercial marine aquariums rely on frozen mullet or baitfish, as a source of food, for resident marine fish, sharks and marine mammals such as seals and dolphins (Dr David Blyde, personal communication, *Sea World*). Studies have shown that *S. agalactiae* can remain viable in frozen wild mullet infected with *S. agalactiae* for up to nine months. *S. agalactiae* has been successfully re-isolated from frozen infected mullet and used in infection trials, causing mortalities in tilapia (Evans *et al*. 2004). *S. agalactiae* infection can be acquired by animals feeding on food contaminated with *S. agalactiae*. For example, a dolphin feeding on wild mullet infected with *S. agalactiae* from a fish kill in Kuwait

succumbed to infection (Evans *et al*. 2006b). Monitor lizards have died from bacterial septicaemia with *S. agalactiae* following ingestion of mice infected with *S. agalactiae* (Hetzel *et al*. 2003). The results from this study should therefore be taken into consideration by veterinarians, recreational and commercial fishermen, commercial marine aquariums (such as *Reef HQ*, *Sea World*), Aquaculture farms, fisheries biologists, researchers, and policy makers when using frozen mullet or baitfish sourced from North Queensland, as a source of food, for captive breeding, cultured or display fish or for other marine animals.

## 7.0 GENERAL DISCUSSION

***Objective 1: To create a library of different S. agalactiae strains enabling utilisation by scientific researchers.***

Ninety six *S. agalactiae* isolates were collected from a diverse array of marine and terrestrial animal species in Australia, from diverse geographical areas within Queensland and the Northern Territory, and were incorporated into a bacterial strain collection currently held at the University of Queensland (UQ) and at Biosecurity Queensland, DAFF. Bacterial isolates of piscine origin include multiple strains from wild sick Queensland grouper, javelin fish, mullet, and catfish. The collection also has multiple marine strains from marine stingrays, saltwater crocodiles, human and terrestrial animals. This strain collection is available for worldwide scientific use, through contact with the Principle Investigator Dr Rachel Bowater Biosecurity Queensland DAFF, or with the project collaborator Associate Professor Andrew Barnes at UQ.

***Objective 2: Develop reliable, rapid and accurate diagnostic tools to enable detection of the bacterial disease streptococcosis caused by S. agalactiae in marine fish.***

***PCR***

A diagnostic polymerase chain reaction (PCR) for use on bacterial cultures from fish with suspect *S. agalactiae* infection was developed and validated, according to the OIE and SCAHLS guidelines. The effectiveness of the PCR as a diagnostic tool, was demonstrated with respect to specificity, sensitivity, repeatability and robustness. *Robustness* of the assay was demonstrated by inter-laboratory PCR (blind testing) done at two independent, Australian State Government aquatic diagnostic laboratories. Both laboratories successfully detected *S. agalactiae* in a panel of ten unknown samples using the specific PCR assay. *Specificity* of the PCR assay to detect *S. agalactiae* on DNA extracts from fish tissues was demonstrated using tissues collected from the injection challenge trail of juvenile Queensland grouper *E. lanceolatus* (Section 6.6). The sensitivity and specificity of the PCR assay on tissues from injected fish, which constitute a "High" Positive sample group, was equal to that demonstrated by histopathology and bacterial isolation from fresh tissue.

Application of the PCR assay to immersion challenge fish that represented 'high', 'medium' and 'low' concentration treatment groups demonstrated good specificity and sensitivity. There was variable agreement between the PCR assay, bacterial isolation and histopathology, likely due to differing sensitivities of the various diagnostic tools, to detect *S. agalactiae*. The PCR demonstrated 100% specificity on all negative fish samples, however it was variably positive with fish samples from various treatment groups. Confirmation of the PCR results was planned to be done, using the fluorescence immunohistochemistry (FIHC) diagnostic tool to further validate the PCR (Section 6.4). The use of the FIHC on fish tissue samples would have enabled further validation studies to be done, allowing sensitivity, robustness and repeatability studies to be completed on fish tissues. Unfortunately, due to closure of the Tropical & Aquatic Animal Health Laboratory (TAAHL) by the Queensland Government, and resultant loss of staff and resources, this work was not able to be done.

***FISH and FIHC***

Two other tests were evaluated as a diagnostic tool to detect *S. agalactiae* in fish tissues, a fluorescent *in situ* hybridization (FISH) based on detection of specific sequences of the 16S rRNA, and fluorescence immunohistochemistry (FIHC) which uses antibodies specific for

antigens at the sub-cellular level on sections of biological tissue. The developed FISH proved unsuitable for the detection of *S. agalactiae* in formalin-fixed and paraffin-embedded (FFPE) fish tissues.

An alternative method, fluorescence immunohistochemistry (FIHC), was developed and optimised using a readily available commercial polyclonal antibody that reacts with type-specific carbohydrate on the surface of Group-B *Streptococcus* (GBS), such as *S. agalactiae*. Detection of this primary antibody was made possible using a commercial secondary antibody, coupled with a photo stable far-red fluorescent dye. The FIHC was specific and highly reproducible and able to be used on formalin-fixed and paraffin-embedded (FFPE) fish tissues. The FIHC detected *S. agalactiae* in fish from the injection, immersion, oral and cohabitation challenge trials. Histopathology undertaken on haematoxylin and eosin (H&E) and Gram-Glynn stains revealed colonies of coccoid bacteria, observed in organs and tissues of infected animals, with accompanying inflammation. The FIHC, when applied to FFPE histological tissue sections (of the same organs), identified the coccoid bacteria as *S. agalactiae*. Control fish negative for *S. agalactiae* by histological examination of Gram Glynn special stain, also tested negative by FIHC. The FIHC allowed good visualisation of the specific location of *S. agalactiae,* in various tissues (similar to that observed by histological interpretation of Gram-Glynn special stains of tissue sections). The FIHC proved to be highly specific and provided confirmatory diagnosis not achieved by routine H&E.

***Objective 3: To perform phylogenetic comparison of Australian fish, human and animal strains of S. agalactiae with overseas fish and animal S. agalactiae strains, to determine their genetic relatedness and origin of the grouper strain (introduced or endemic)***

The complete genomes of 23 *S. agalactiae* isolates were sequenced and a preliminary assembly completed. Final assemble and full annotation is ongoing. From the assembled genomes, the isolates were typed by multilocus sequence typing (MLST) and serotyped. MLST revealed that all of the piscine isolates of *S. agalactiae* from Australia fell into the strain type, ST-261 group. This is a one of a number of related strain types that are commonly found in fish (Evans *et al*. 2002, 2008 & 2009; Lusiastuti *et al*. 2009ab, 2014). Amongst the fish sequence types, ST-261 has only been identified previously in tilapia, and is identical to the strain type that was recovered from Nile tilapia (*Oreochromis niloticus*) in Israel and subsequently translocated to the US and Brazil (Evans *et al*. 2008). This is intriguing, since of all of the fish sequence types occurring globally, only ST-261 is present in Australia and has also very recently been reported from Indonesia, a strain type associated with Nile tilapia (Lusiastuti *et al*. 2009ab, 2014). Tilapia and hybrid tilapia species have been introduced into North Queensland on several occasions since the 1970s, around Cairns and Townsville regions and several species and hybrids are present in many Northern rivers (Mather & Arthington, 1991). It is possible, that this strain type was introduced along with tilapia, and has since established amongst the Australian aquatic animal life in the region.

One new MLST allele was discovered in a grouper bacterial isolate. As the genes used for MLST occur in the core genome and are under positive selective pressure, they evolve very slowly. At this time we are unsure of mutation rates in *S. agalactiae*, but a single allele change amongst the ST-261 strain type suggests a relatively recent introduction, as there has been insufficient time for major allelic variation.

The preliminary whole genome sequence comparison and *cps* sequence typing corroborate the MLST. The marine isolates were not closely related to terrestrial animal or human

isolates (the genomes are 200-300 kb smaller) and have therefore not been recently transferred from terrestrial sources. This is reassuring from a public safety perspective as these sequence types have not been found causing disease in humans or terrestrial animals. They are therefore unlikely to pose a food safety issue, as these sequence types occur in food fish overseas and have not been associated with human illness.

The substantial gene loss amongst the marine isolates may include loss of genes essential for colonization of mammals, although this will require complete annotation and further analysis of the genomes.

The *cps* genotyping provided some further resolution of possible strain movement and evolution within the aquatic animals. Identical point mutations have been found in all of the isolates from stingrays suggesting passage amongst these animals. Moreover, four of the grouper isolates, the javelin fish and the catfish isolates, shared identical *cps* genotype. Bearing in mind the plasticity of the *cps* operon in *Streptococcus* spp., this strongly implicates a shared source of infection amongst these fish. It is possible, that the catfish and javelin fish obtained infection with *S. agalactiae*, by feeding on dead grouper carcasses, or vice versa, or that all three different fish species (all were found sick in Trinity Inlet, Cairns), were feeding on a common source of infection. This requires further investigation.

***Objective 4: Perform a challenge infectivity trial in Queensland grouper to prove experimentally that S. agalactiae causes mortalities in Queensland grouper to fulfil Koch's postulates.***

The results from the four experimental challenge trials, effectively demonstrated that juvenile Queensland grouper *E. lanceolatus,* can become infected with *S. agalactiae* by; injection with the bacteria; co-habitation of healthy fish with infected fish; water-borne exposure; or ingestion of infected food. These experiments effectively fulfilled Koch's postulates, and proved that *S. agalactiae* causes illness, gross clinical signs of streptococcosis and mortality in Queensland grouper.

The combined laboratory test results (from bacteriology, PCR and histopathology) from the injection challenge trial, confirmed that fish injected with *S. agalactiae* became infected and developed overt clinical signs and pathology typical of streptococcosis by infection with *S. agalactiae*, as reported in the literature (Abuseliana *et al*. 2011; Amal *et al*. 2012; 2013; Chen *et al*. 2007; Evans *et al*. 2006a; Filho *et al*. 2009; Evans *et al*. 2014). This experiment effectively demonstrated that the *S. agalactiae* bacterial strain QMA0825 (originally isolated from the kidney of a wild Queensland grouper that died in Townsville in 2010), is highly virulent to juvenile grouper *E. lanceolatus*. When injected into fish (at various dose rates) fish developed clinical signs of streptococcosis and died within three to four days, with nearly 100% mortality. There was a marked dose effect, with fish injected with higher doses developing more severe and marked pathology.

The combined laboratory test results from the immersion challenge trial, (where fish were immersed for an hour in seawater containing three different doses of *S. agalactiae)*, developed a milder inflammatory response, and developed infection at a much slower rate, and all (except one fish) survived the experiment. Fish showed no overt clinical signs of infection apart from lethargy and short-lived anorexia, but with recovery, and many were positive by PCR, bacteriology or pathology for *S. agalactiae*. This experimental trial effectively demonstrated that *S. agalactiae* can spread through the water and infect juvenile

grouper, regardless of the exposure dose. Special histological stain (Gram-Glynn) and application of the FIHC to formalin-fixed, paraffin embedded sections, enabled visualisation of Gram-positive cocci at the cellular level, in multiple organs and tissues of the surviving fish. This experiment provided evidence that juvenile grouper exposed to the bacterium in the water, can survive and act as subclinical carriers of *S. agalactiae*.

Results from both the oral and cohabitation challenge trials, indicated the transmission routes for *S. agalactiae* in juvenile Queensland grouper *E. lanceolatus,* may occur via the oral route (ie. by ingestion of infected food) or by cohabitation of a healthy fish with an infected fish (ie. via water-born route). The combined laboratory test results (PCR, histopathology and bacteriology, gross pathology and observed clinical signs) for both challenge trials, and the fact that most fish survived for the five week duration period (for both experiments), indicated most surviving fish were either subclinical carriers of *S. agalactiae* (showing little or no overt signs of bacterial infection), or were at the early stages of infection and only just starting to show signs of streptococcal infection. Further experimental challenge trials, extending the duration of the experimental challenge period would be necessary to confirm this.

In the oral challenge trial, the combined laboratory results (PCR, bacteriology, histopathology analysis, observed clinical signs and post mortem findings) showed that juvenile grouper *E. lanceolatus*, fed with both high and low doses of *S. agalactiae*, can become infected with *S. agalactiae* via the oral route. Laboratory results showed there was a very low level of infection of experimental fish with *S. agalactiae*, for both replicate systems. All fish (except two) survived for the entire five-week duration of the experiment, indicating most surviving experimental fish were infected with *S. agalactiae* but were subclinical carriers. These fish had full stomachs on dissection after euthanasia, indicating the fish had been eating. Two fish from the high dose treatment group became moribund, and showed clinical signs of streptococcosis including darkening, loss of appetite, separation from the cohort, spiral swimming, unilateral exophthalmia, ascites, and reddening of the caudal or pectoral fins, and empty stomachs.

In the cohabitation challenge trial, the combined laboratory results (PCR, bacteriology, histopathology analysis, gross clinical signs and post mortem findings) showed that healthy, non-infected juvenile grouper *E. lanceolatus*, cohabited with juvenile grouper injected with *S. agalactiae*, can become infected with *S. agalactiae* by cohabitation within the same tank, or in a different tank, but within the same recirculation system (despite biological filtration, and a protein skimmer). Only four fish died during the five week duration of the experiment, and a further seven fish were moribund at the end of the five week experiment (six of these seven fish were from the injected treatment groups, and one fish was from a non-injected treatment group). Infected moribund fish showed clinical signs of streptococcal infection including anorexia, spiral swimming, unilateral exophthalmia, unilateral corneal opacity, hyperaemia of the caudal or pectoral fins, splenomegally, a reduced sized liver or spleen, but no petechial haemorrhages on the gills or vents (as seen in fish from the injection challenge trial). Most surviving fish had stomachs that were a third-half full or completely full, indicating they had been eating.

Comparison of all experimental challenge trials, showed that the onset of infection of streptococcosis for juvenile grouper differed, depending upon the route of exposure of fish to *S. agalactiae*. The onset of infection was fastest for fish injected intra-peritoneally with *S. agalactiae*, with clinical signs and death occurring quickly on days 2 to 3 post-injection.

These results are similar to other transmission trials conducted on other fish species, challenged by injection with *S. agalactiae* (Evans *et al*. 2002; Filho *et al*. 2009; Geng *et al*. 2012; Mian *et al.* 2009; Musa *et al*. 2009; Suanyuk *et al*. 2008). In comparison, the oral route of infection was much slower, with the onset of disease becoming apparent on day 26 and day 33. The progression of disease for juvenile grouper with *S. agalactiae* by cohabitation was slower, with fish initially becoming moribund on day 10, but then fish were observed to recover and most went on to survive the five weeks of the experiment.

Statistical comparison and analysis of all three laboratory test methods to detect *S. agalactiae* in fish tissues, from both the oral and cohabitation challenge trials showed differing test results, but PCR was found to be the most sensitive test method.

Histopathology analysis was found to be a useful tool for visualising the infection process and enabled a diagnosis to be made on an individual fish basis, based on histological examination and observation of the disease process in all organs and tissues within a fish. Furthermore, bacteria could be visualised at the cellular level in multiple organs and tissues. Histopathology in Queensland grouper with streptococcosis from infection with *S. agalactiae* was consistent with that described in other fish species (Evans *et al*. 2004; Filho *et al*. 2009; Evans *et al*. 2014). Interestingly, some pathology was observed in fish from this project that has not been described or reported previously from fish infected with *S. agalactiae*.

Detailed histological analysis (done on Gram-Glynn special stain of fish tissues), of fish from the injection, immersion, cohabitation and oral challenge trials, enabled detection of *S. agalactiae* at the cellular level, in cells within multiple organs and tissues. Histological observations showed the presence of *S. agalactiae* observed inside intact fish tissue macrophages, and often in phagocytic vacuoles. From these microscopic observations, it is reasonable to speculate that the bacterium may be able to remain intact (once engulfed by macrophages), inside tissue macrophages, and in this way, cross the blood brain barrier undetected, and gain entry into the brain of host fish. It is possible the bacterium may interfere with normal apoptosis following phagocytosis, either inducing apoptosis, or halting or prolonging programmed cell death, thus enabling on-going survival in the fish host and thus be transported around the body. This is similar to what has been described for *S. iniae* pathogenesis in fish, ie. the '*Trojan horse effect'*, whereby the bacterium is able to be transported around the body undetected by the hosts' innate immune system (Zlotkin *et al*. 2003). Further research is required to investigate and test this hypothesis. Nonetheless, it is evident from light microscope and histological observations that the macrophages of juvenile Queensland grouper play a crucial role in the innate immune response of juvenile Queensland grouper to the Gram-positive cocci bacterium *S. agalactiae.*

***Objective 5: To determine the potential food source of infection for Queensland grouper in Trinity Inlet, Cairns.***

Surveillance study results indicated the prevalence of *S. agalactiae* in wild fish and crustaceans in Trinity Inlet, for the period September 2010 to April 2012 was at levels which are undetectable, using current available diagnostic methodology (PCR and bacteriology). *S. agalactiae* was not detected from a total of 522 potential prey items sampled from Trinity Inlet, Cairns, from September 2010 to April 2012. Prey items tested included; 126 fish (comprising eleven different species); 106 baitfish (<30 mm TL, comprising seven different species), 100 tilapia (*Oreochromis mossambicus*), collected from suburban storm water

drains in Cairns, (that drain into Trinity Inlet); one stingray (*Rhinoptera javanica*) and189 crustaceans (105 mudcrabs *Scylla serrata*, and 84 banana prawns, *Penaeus merguiensis*).

A further 268 marine animals (fish and crustaceans of various species) sampled from Cairns from September 2010 to September 2012 (section 6.9), all tested negative for *S.agalactiae.*

The future development and application of more sensitive diagnostic tests (such as qPCR), in field surveillance studies, targeting potential carrier species of *S.agalactiae*, may shed further light on the potential source of infection of *S. agalactiae* for Queensland grouper.

***Objective 6: To determine the distribution and prevalence of <u>S. agalactiae</u> in fish along the North Queensland coast***

***(a) Targeted surveillance***
*S. agalactiae* was not detected using current available diagnostic methodology (PCR and bacteriology), from targeted surveillance of 179 wild, healthy mullet, from four different geographical regions along the Queensland coast, including; Weipa (in the Gulf of Carpentaria), Cairns, Townsville and Proserpine (on the eastern coast) of Queensland.

***(b) Extra samples tested***
*S. agalactiae* was not detected, using current available diagnostic methodology (PCR and bacteriology) from an extra 363 wild marine species (153 marine fish, 179 crustaceans and 10 freshwater/brackish-water fish) tested for *S. agalactiae*. These samples spanned a coastal area covering over 3300 km, from as far south as Bundaberg, to Weipa. Fish sampled included diseased fish from fish kills in Weipa, Lakefield National Park, Gladstone and Rockhampton. Fish were also sampled from regions where dead Queensland grouper were previously reported including; Bundaberg, Gladstone, Rockhampton, Proserpine, Bowen, Ayr, Townsville, Cairns, Port Douglas and Weipa (Bowater *et al.* 2012*).*

Two fish had suspect, unconfirmed positive PCR results for *S. agalactiae*; an eeltail catfish *(Neosilurus* sp.) from a large-scale fish kill (involving multiple fish species), that occurred in the Normanby River near Lakefield National Park; and a marine mullet (*Mugil cephalus*) sampled from Gladstone. Both fish had weak positive amplicons from brain tissue sampled by PCR. Bacteriological sampling of the brain of the catfish yielded a *Streptococcus* sp. but this was not confirmed as *S. agalactiae*. The eeltail catfish also had a bacterial septicaemia from infection with the bacterium *Aeromonas sobria* and was concurrently infected with whitespot disease. It is presently unknown if the PCR result represented a significant finding, or was a result of non-specific amplification, since post mortem degradation of tissues occurred due to the remote location and inability to obtain fresh material for PCR and bacteriology (fish was frozen prior to sampling). The mullet sampled from Gladstone, was a freshly caught, wild fish, and the brain was preserved in ethanol for PCR analysis. The unconfirmed PCR results for both the mullet and eeltail catfish may indicate possible asymptomatic carriage of *S. agalactiae*. These results warrant further targeted surveillance of sea mullet (*Mugil cephalus*) and eeltail catfish (*Neosilurus* sp.), and related fish species, with application of more sensitive diagnostic tests for *S.agalactiae.*

In Australia many large commercial marine aquaria (such as *Sea World*) rely on frozen mullet or baitfish as food for resident marine fish, sharks and marine mammals such as seals and dolphins (D. Blyde, personal communication, *Sea World*). *S. agalactiae* can remain viable in frozen wild mullet infected with *S. agalactiae* for up to 9 months and *S. agalactiae* has been

successfully re-isolated from frozen infected mullet and used in infection trials causing mortalities in tilapia (Evans *et al*. 2004). *S. agalactiae* infection can be acquired by animals feeding on food contaminated with *S. agalactiae*. For example, a dolphin feeding on wild mullet infected with *S. agalactiae* from a fish kill in Kuwait succumbed to infection (Evans *et al*. 2006b) and monitor lizards have died from bacterial septicaemia with *S. agalactiae* following ingestion of mice infected with *S. agalactiae* (Hetzel *et al*. 2003). Results from this project indicate frozen and fresh baitfish (mullet) should be considered a low risk as a potential source of infection of *S. agalactiae* for wild grouper or other wild fish species in Queensland. However further sampling of wild mullet and other baitfish species, using more sensitive PCR/ other tests with test capability to detect carrier status, would be useful to elucidate further the potential sources of infection for Queensland grouper and other native fish species.

# 8.0 CONTACT WITH BENEFICIARIES

An article on dead groupers relating to this research was mentioned under '*short takes'* in the *Ghostnets Australia Newsletter* Issue 5.

Results from this research project were presented to members of the National Scientific Aquatic Animal Health community at the 1st Australasian Scientific Conference on Aquatic Animal Health in Cairns, in 5-8 July, 2011 and at the 2nd Australasian Scientific Conference on Aquatic Animal Health in Cairns on 8-12 July, 2013.

Results from this project were also presented at the Ridleys Australian Barramundi and Prawn Farmers' Association annual industry meeting held in Palm Cove, July 31-August 2, 2013.

The principal investigator was invited to speak about the Queensland grouper deaths at the Townsville Local Marine Advisory Committee (TLMAC) meeting, chaired by the GBRMPA (Great Barrier Reef Marine Park Authority), and attended by local Towsnville Deputy Councillor Vern Veitch, DERM, GBRMPA and other local authorities at Townsville in August, 2010.

A fact sheet was produced by the Queensland Grouper Mortality Investigation Task Force in 2009, in conjunction with the GBRMPA and the PI (Rachel Bowater) of Bioseurity Queensland, DAFF (Appendix 12). This fact Sheet was produced to increase awareness about dead and dying Queensland grouper, providing relevant information on seafood safety and to assist with reporting of dead or dying wild Queensland grouper, and to therefore also assist the PI with bacterial strain collection (objective 1) of this research study. This Fact Sheet was posted on the world wide web and was posted at fishing shops in the Townsville and Cairns regions.

Radio interviews were conducted and aired, about this research, on both local and National ABC Radio in 2010, 2011 and 2012.

# 9.0 BENEFITS AND ADOPTION

The general public at large has benefited from the knowledge that the deaths of wild Queensland grouper in highly urbanised areas was attributed to natural infection with *S. agalactiae*, and that the source of infection for Queensland grouper was highly likely to be from natural causes. It is highly unlikely that Queensland grouper acquired infection with *S.agalactiae* from ingestion of contaminated sewer effluent, hospital effluent or effluent arising from dumps adjacent to coastal waterways where Queensland grouper frequent eg. in Trinity Inlet and associated tributaries of Cairns, North Queensland.

GBRMPA (the Great Barrier Reef Marine Park Authority) has benefited with scientific knowledge gained from this project that showed *S. agalactiae* is present at nearly zero prevalence in fish, mudcrab and crustacean populations in Trinity Inlet in Cairns and in other coastal areas of North Queensland including Townsville, Bowen, Proserpine, Mackay, Gladstone, and Rockhampton indicating *S. agalactiae* presents a very low risk to natural fisheries resources and other marine species within the Great Barrier Reef Marine Park. The risk of overall impacts of *S. agalactiae* on the social amenity of the Great Barrier Reef and direct impacts on the economics of the industries that rely on this ecosystem including recreational and commercial fisheries are considered low.

Aquaculture farms, marine fish breeding and culture facilities, commercial marine aquariums and the ornamental fish trade have all benefited, and will benefit in the future from the scientific knowledge on *S. agalactiae* generated from this project including; transmission pathways of *S. agalactiae* in grouper, the development of diagnostic tools to detect *S. agalactiae* in disease outbreaks in Queensland grouper (or other cultured grouper species) and future research potentially arising from this project, including health and disease management strategies, biosecurity protocols, and possible vaccine production, in relation to the prevention, control, treatment and eradication of *S. agalactiae* in fish farms and marine fish display aquariums. This will lead to improved fish health and disease management, and promotes ongoing sustainability of both aquaculture and natural fisheries resources.

The general public, the seafood industry, retail bait outlets, commercial and recreational fishers, *Sea World* staff and other commercial display aquariums have benefited from scientific knowledge that *S. agalactiae* found in dead wild Queensland grouper is unrelated to human strains of *S. agalactiae* and therefore is unlikely to be of significant zoonotic risk to people handling dead or sick Queensland grouper, stingrays, or other fish species infected with *S. agalactiae*. Surveillance studies showed zero prevalence in mudcrabs, banana prawns and a variety of fish species (including frozen baitfish) in Cairns, Townsville, Proserpine and Weipa, giving the public and these groups of people, consumer confidence in handling and consuming seafood and handling bait sourced from these coastal regions.

The scientific community in Australia and worldwide has benefited from the collection of a library of 96 bacterial isolates of *S. agalactiae* held at both UQ and DAFF, that can be utilised for future scientific research on various genetic, immunological, and pathogenicity studies on *S. agalactiae*, for both marine fish, animal and human studies.

The State and Commonwealth veterinary laboratories, University laboratories, government agencies and DAFF Australia, have benefited from the development of diagnostic tools that can be utilised for improved disease detection, reporting and monitoring of *S. agalactiae* in

both wild fisheries resources, aquaculture enterprises, the marine aquarium trade and commercial marine aquariums.

Contact and liason with recreational and commercial fisherman, indigenous communities and the general public in North Queensland coastal regions during sampling trips was beneficial and rewarding. The production and dissemination of a fact sheet, dissemination of information to the scientific community, to local marine advisory groups, to council and via newsletters and workshops with GBRMPA and DERM was of benefit, resulting in increased awareness of the disease in Queensland grouper and seafood safety in the general public, to commercial and recreational fishing communities, indigenous fishing communities, the tourism industry, the seafood industry, along the east coast, and in the Gulf of Carpentaria of Queensland during the course of this study.

# 10.0 FURTHER DEVELOPMENT

This project was successful in demonstrating that the emerging bacterium *S. agalactiae*, isolated originally from wild dead Queensland grouper *Epinephelus lanceolatus* in North Queensland Australia, experimentally reproduced streptococcal disease when the same bacterium *S. agalactiae* was injected into juvenile Queensland grouper, resulting in clinical signs of streptococcosis, brain infection, bacterial septicaemia and death. The project demonstrated, through several other experiments, that *S. agalactiae* can be spread from infected grouper, to uninfected grouper, by co-habitation (of infected and non-infected fish) both within a tank, and between tanks, in a marine recirculation system; that juvenile grouper can become infected with *S. agalactiae* by ingestion of feed contaminated with *S. agalactiae*, or by immersion of fish in water contaminated with *S. agalactiae*.

The project was successful in showing that bacterial isolates of *S. agalactiae*, obtained from wild, dead adult Queensland grouper, were genetically distinct from human, or other animal *S. agalactiae* isolates, and therefore it is unlikely Queensland grouper acquired infection from ingestion of food or water contaminated with human effluent (eg. seepage into local waterways from hospital waste or sewer effluent) or from other animal effluent (e.g contaminated waste water discharged from catteries, dog kennels or crocodile farms).

The surveillance studies yielded some results warranting further scientific investigations. Two species of wild native fish with unconfirmed weak positive PCR results for *S. agalactiae;* a sick eeltail catfish *(Neosilurus* sp.) from a wild fish kill, and a healthy marine mullet (*Mugil cephalus*) sampled from Gladstone. These results also highlight the importance of the continuation of multi-agency fish kill investigations, with disease investigation done by aquatic animal health specialists, since infectious disease must be considered as a possible cause of death in any wild fish kill event, in any jurisdiction of Australia.

Recommendations for further development include;

1. The utilisation of knowledge gained from this project for the development of specific farm health and disease management plans (FH&DMP's) in relation to the prevention and control of *S. agalactiae* on aquaculture farms and commercial display aquariums. Further research on specific aspects of the pathogen, including the effect of various disinfectants/biocides on pathogen viability/survival, in water, on biofilms, with field trials done in aquatic enterprises, would aid in development of specific biosecurity protocols for *S. agalactiae* control for the aquaculture industry. Biosecurity protocols could be tailored or refined as necessary, to meet the specific needs of various aquaculture farms. This is particularly relevant for aquaculture facilities where wild Grouper are relied upon as broodstock for purposes of aquaculture production (eg. Sunfish Enterprises, Cairns), and for commercial tourism operators where disease outbreaks from *S. agalactiae* have already occurred (eg. *Sea World*, south east Queensland). Development of FH&DMP's are critical to prevent and control streptococcal disease outbreaks on farms, thus promoting future sustainability of both the aquaculture industry and natural fisheries resources, both within and outside the Great Barrier Reef Marine Park.

2. The development of challenge models in barramundi, other high-value grouper species and other Australian cultured tropical and temperate fish species, to determine

the potential susceptibility of these native Australian fish species to *S. agalactiae,* and to determine possible routes of transmission. This information is critical to determine the risk that *S. agalactiae* poses to the Australian aquaculture industry as a whole, and is relevant to all jurisdictions. Multiple fish species are cultured in all states of Australia, and *S. agalactiae* has been shown to cause disease epidemics in a wide range of fish hosts (worldwide), in both cultured and wild fish species. Outcomes from this work will also support the development of on-farm health and disease management plans (point 1), further promoting sustainability of the Australian Aquaculture industry and natural fisheries resources, into the future.

3. Further targeted surveillance of specific Australian native fish and elasmobranch species shown to be infected/susceptible to *S. agalactiae* (mullet, javelin fish, fork-tailed catfish, stingrays, barcoo grunter), introduced feral fish species known to be susceptible to *S. agalactiae* in overseas countries (eg. tilapia, hybrid tilapia), and fish with unconfirmed PCR-positive results for *S. agalactiae* (eeltail catfish, marine mullet) to elucidate the potential source of infection for wild Queensland grouper. This would be done using more sensitive diagnostic tests (below), to test whether these or other closely-related fish species are subclinical carriers of *S. agalactiae*, using a multi-agency approach for investigation of fish kill events.

4. The development and application of more sensitive scientific diagnostic tests (such as qPCR), to be used for above mentioned surveillance studies.

5. Further scientific laboratory studies including utilisation of the FIHC diagnostic tool to further validate current diagnostic methodologies developed in this project (namely histology, bacteriology and PCR), and for further development of more sensitive PCR tests, would be useful to further strengthen the current diagnostic tools used to detect *S. agalactiae* in fish.

6. Policy revision and development of State policies regarding documentations for inter-state movement of marine animals, health testing requirements and quarantine upon arrival, of wild-caught marine animals, to be utilised for the purposes of aquaculture or display purposes, are needed prior for both intra-state and inter-state translocation in Australia. This is based on current knowledge of at least 2 outbreaks of streptococcosis (from infection with *S. agalactiae)*, which occurred at *Sea World* in 2009 and 2010. Both outbreaks occurred after wild-caught stingrays of unknown disease status, were translocated from North Queensland to South-east Queensland. Policy development should include an appropriate record of numbers and types of animals moved around, to allow for traceability (both forwards and back), and include adequate records, a suitable quarantine and acclimation period for tranlocated stock, and health assessment of stock by a qualified aquatic veterinarian, and possibly diagnostic testing, prior to and post-shipment.

7. Further genetic, immunological and molecular studies to determine likely virulence factors contributing to the pathogenicity of *S. agalactiae* in Queensland grouper, other grouper, fish and elasmobranch species may be useful information to assist and underpin any future vaccine studies in the future. Steering away from antibiotic use and use of vaccines is farm will promote on-going sustainability of both the aquaculture industry and wild fisheries resources within Australia.

# 11.0 PLANNED OUTCOMES

The overall planned outcome of this project was to assist the sustainability and health of natural resources and the profitability of the aquatic industry. This has been achieved by providing the public, industry, governments, and the private sector with knowledge on the cause of deaths of over 96 wild adult Queensland grouper in eastern coastal Queensland and the Gulf of Carpentaria, the transmission routes of *S. agalactiae* in Queensland grouper, and the prevalence of *S. agalactiae* in wild fish and crustacean populations along the east coast of Queensland, Australia.

A planned outcome of this project was increased State and National fish disease reporting and monitoring, and the development of health and disease management plans (in relation to *S. agalactiae*), utilised by the Aquaculture and marine aquarium industry, in all jurisdictions of Australia. This was achieved through the development of knowledge and diagnostic tools facilitating future streptococcal disease detection, enabling disease reporting and monitoring; development of on-farm biosecurity, health and disease management plans; and future policy development in relation to translocation of wild caught, marine animals (in relation to *S. agalactiae*), for use by aquatic enterprises and governments, in all jurisdictions of Australia. This helps protect recreational fisheries through improved translocation policies, improved disease management on Aquaculture and marine aquarium facilities, and thereby assists in the conservation of natural fisheries resources, including threatened marine finfish species of the GBRMP, allowing improved management of the GBRMP, and helping to promote aquaculture.

A broader outcome to the Australian community was increased seafood consumption. This was achieved through molecular, genetic studies that clearly illustrated the Queensland grouper *S. agalactiae* isolates were genetically distinct from human and other animal isolates; and from surveillance studies that demonstrated absence of *S. agalactiae* in wild fish and crustacean populations along the east coast of Queensland.

A further outcome of this project was increased scientific research on *S. agalactiae*. This was achieved through the provision of a library of bacterial isolates of *S. agalactiae*, available at both UQ and QDAFF.

## 12.0 CONCLUSION

This project was successful in demonstrating that the emerging bacterium *S. agalactiae*, which was responsible for killing numerous adult, breeding sized wild Queensland grouper *E. lanceolatus* in North Queensland Australia, between 2007 and 2012, is pathogenic to juvenile Queensland grouper. Four separate experimental fish challenge trials, demonstrated that *S. agalactiae* is pathogenic to juvenile Queensland grouper, and that *S. agalactiae* can spread via contaminated food, water, or from an infected to uninfected fish; within the same tank, or between tanks, in a recirculation system, despite physical separation of tanks and the presence of a biological filtration system and a protein skimmer. Experimental trials showed the onset of clinical signs of streptococcal disease differed, depending upon the route of exposure of fish to *S. agalactiae*. Fish injected with *S. agalactiae* developed clinical signs of streptococcal infection and died within 2 to 3 days. Fish infected via the oral route, developed clinical signs on day 26 to 33, recovered and survived as subclinical carriers (as evidenced by PCR & histopathology). Fish infected via co-habitation (a healthy un-infected fish living in close proximity to an infected fish) became moribund on day ten, recovered, and survived as subclinical carriers (as evidenced by histopathology & PCR). Both the oral and cohabitation challenge trial experiments demonstrated that juvenile Queensland grouper can become infected, recover, and survive infection with *S. agalactiae,* as subclinical carriers.

This project demonstrated, through specific molecular studies of nucleic acid sequence analysis, that multiple bacterial isolates of *S. agalactiae* (isolated from multiple dead wild Queensland grouper), belong to a common piscine strain type, ST-261, a strain type that is genetically unrelated to human, crocodile, dog, cat or other farm animal strain types that exist in Australia. It is therefore unlikely that wild Queensland grouper acquired *S.agalactiae* infection from anthropogenic sources, and is unlikely to present a zoonotic risk. This is reassuring for the general public, seafood industry and seafood consumers.

Molecular studies done in this project, further showed that the *S. agalactiae* isolates obtained from wild, dead, Queensland grouper, were most closely related to the isolates of *S. agalactiae* isolated from javelin fish, mullet, catfish and stingrays from North Queensland, all belonging to strain type ST-261. This particular strain type, ST-261, has been identified from Nile tilapia and hybrid tilapia species during disease outbreaks in Indonesia, China, Brazil, Israel and other overseas countries, that intensively culture tilapia as a food source. A number of tilapia, and hybrid tilapia species were introduced into North Queensland by aquarists over 30 years ago (Arthington *et al*. 1984). Tilapia are a declared noxious feral pest in Queensland, having successfully invaded local waterways, established breeding populations in dams, creeks, rivers and local waterways, displacing many native fish species from their natural habitats, competing for food, and have spread South, North, East and West of Cairns. The very close genetic relationship of the Qld grouper *S. agalactiae* strain to the tilapia strain from Israel and the USA is intriguing, and further research on the genomes of these strains is necessary and ongoing.

The project demonstrated, through targeted surveillance and sampling of over 1300 wild fish, that *S. agalactiae* was not detected (using current available diagnostic methods) in wild fish and crustaceans in Trinity Inlet, Cairns, and other coastal regions of Queensland, between 2010 and 2012. *S. agalactiae* therefore poses a very low risk to natural fisheries resources, and to fish within the Great Barrier Reef Marine Park. This is also reassuring for the GBRMPA Managers, as *S. agalactiae* likely presents a low risk to threatened and vulnerable marine species, such as marine mammal cetacean species, dugongs and turtles. This is

supported by molecular genotyping that demonstrated the strains generally infecting marine mammals are generally from clonal complexes normally associated with human infection, and only very distantly related to those infecting wild fish in Australia.

This project demonstrated, through scientific sampling of over 200 frozen baitfish and mullet, that *S. agalactiae*, was not detected, (or is present at such a low prevalence as to be undetectable) in frozen baitfish using currently available diagnostic tools and sampling strategy. However, we must be mindful that we have previously isolated *S. agalactiae* from a mullet species occasionally sourced as commercial baitfish from Trinity Inlet, in Cairns (Bowater *et al*. 2012). Frozen baitfish therefore presents an unknown risk as a potential source of infection to Queensland Grouper and other wild fish. However further modified sampling of wild mullet and related species (as previously indicated), should be considered to better elucidate this potential risk.

This is also important in light of the continued reliance of many large commercial marine aquariums, such as *Sea World* or *Reef HQ,* on frozen mullet (baitfish) as a source of food for their display animals. Two large disease epizootics from *S. agalactiae* hve already occurred at *Sea World* in 2009 and 2010, with an as yet unidentified source of infection. Although translocation of an infected stingray from North Queensland is currently suspected, genotyping cannot rule out infected mullet as a potential source. The implications of these observations should be considered by state and national aquatic animal health policy makers, in regards to translocation of wild-caught marine animals of unknown health status, and in regards to health testing of wild-caught baitfish.

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# APPENDIX 1. INTELLECTUAL PROPERTY

**This project has not developed any intellectual property that requires legal protection.**

# APPENDIX 2. STAFF LIST

Many people contributed to the research in this project. The core project team included Rachel Bowater (Principle Investigator), Andrew Barnes (co-investigator), Jerome Delamare-Deboutteville (PhD student), Andrew Fisk, Kelly Condon and Kerry Dyer. The PI, Rachel Bowater would like to personally thank all the following people who contributed to the work done in this project in some or many ways;

**Associate Professor Andrew Barnes,** University of Queensland provided valuable microbiological and molecular scientific contribution, including supervision of PhD student, Mr Jerome Delamare Deboutteville for the 3 year duration of this project.

**Mr Jerome Delamare-Deboutteville** (PhD student, University of Queensland) worked under the supervision of Associate Professor Andrew Barnes, UQ, on several aspects of the project including running the experimental challenge trials, sampling fish for bacteriology, histology PCR, molecular phylogenetics, development of the FIHC test, and other aspects of fish immunology for his PhD (not covered in this project). Jerome is thanked immensely for his great efforts and enthusiasm.

**Mr Andrew Fisk** Senior Technical officer, Biosecurity Queensland, Department of Agriculture, Fisheries & Forestry (DAFF) worked on many aspects of the project for over three years, including advising, and assisting UQ with improvement and upgrading of aquaria and water quality at the UQ facility, for the running of four experimental challenge trials, and assisted Jerome with acclimatising juvenile Queensland grouper on arrival at UQ, prior to the first experimental challenge trials. Andrew assisted with all field work aspects of the fish surveillance activities, organised and ran numerous field trips, supervised Kerry Dyer on field trips, and caught (with the assistance of commercial fishers and NFC staff) over 1300 wild fish from the Gulf of Carpentaria and East coast of Queensland, from multiple study sites in Northern Queensland, (including Gladstone). Andrew has the rare ability to work with people of different backgrounds, including recreational and commercial fishermen, laboratory staff, researchers and veterinarians. Andrew also provided invaluable assistance with microbiological sampling of all fish collected for surveillance purposes and assisted with various laboratory tasks including data entry, histology, microbiology and PCR.

**Mrs Kelly Condon** developed and conducted validation studies on the PCR at TAAHL, supervised and conducted over 2000 PCRs for all Queensland grouper tested, from all four experimental challenge trials and ran PCR's on nearly 6000 fish organ samples collected from the field surveillance studies. Kelly also supervised laboratory technician Stacey Valdeter, coordinated and organised inter-laboratory testing of the PCR with 2 other State Veterinary laboratories, and worked professionally and tirelessly, with accuracy and zeal (despite the adversity of TAAHL closing in 2012) for the duration of the 3 year project. Kelly is thanked greatly for her huge contribution to this project.

**Miss Kerri Dyer** worked as the part-time project technician, for nearly 2 years, before heading to JCU for full-time work. Kerri worked enthusiastically in the field assisting Andrew Fisk with fish capture, and in the laboratory assisting with wild Grouper post

mortems, cutting in some experimental fish tissues for histological processing, and assisted with microbiology. Kerri also conducted some data entry and used Corel graphics and is thanked for all her work.

**Mrs Jenny Stanford** was the histologist on the project, and is thanked for her hard work and patience in sectioning, staining and mounting over 3000 fish slides, including special staining techniques, and her great sense of humour throughout the entire duration of the Project kept us in good spirits.

**Mrs Angela Anderson** and **Mr Bob Meyer** provided statistical advice and assisted with statistical analyses, and their valuable contribution is greatly acknowledged.

**Mrs Charlotte Williamson (BQ Veterinary Officer, DAFF)** updated the original database of dead and dying grouper from 2007, and her efforts are greatly appreciated.

**Mr Paul Hickey** and **Mrs Sue Everingham (DAFF),** both provided external project financial admnsitrative guidance and their support and great sense of humour are greatly appreciated.

**Mrs Melanie Turnbull** (administrative assistant) provided great in-kind administrative support during the entireity of the project and was a hard-working and valued member of the project team.

**Mr Tim Lucas** (QDAFF) assisted with editing, and provided comments on the report, and this help was greatly appreciated.

**Mrs Judy Forbes-Faulkner** is thanked for her invaluable expertise in microbiology and in identifying the original *S. agalactiae* isolate obtained from the first wild dead Queensland grouper we necropsied at TAAHL. Judy also assisted me with several post mortems done on numerous other dead wild Queensland grouper carcasses at TAAHL, and willingly assisted training the project technician, Kerri Dyer, with all microbiological aspects of the project.

**Mrs Naomi Hooper** worked on the project for 2 weeks, prior to obtaining full-time work elsewhere, and is thanked for her expertise in microbiology, organisational skills and familiarity with TAAHL procedures.

**Helen Smith** is thanked for her assistance with all aspects of the project including assisting the PI with wild Queensland grouper post mortems, registration of samples, microbiology, and PCR.

**Stacey Valdeter** carried out hundreds of PCR's assisting Kelly, and with PCR validation & testing experiments and is thanked for her conscientious and diligent work and great culinary skills (appreciated by all staff at tea breaks).

**Dr Nouri Ben Zakour** (UQ) is thanked for writing the bioinformatics pipelines for cleaning and assembling Illumina sequence data, for extracting the MLST and CPS operon data from the resulting genomes and for determining serotype of the isolates in this study.

**Mrs Virna Duffy, Mrs Liz Kulpa, Mr Bill Doherty, Mr Chris Wright, Mrs Rafidah Jamaludin, Dr Ian Anderson, Dr Annette Thomas** assisted with various task such as grouper post mortems, mapping, histology and microbiology aspects of the project.

**Dr Cathy Shilton** kindly provided crocodile isolates from Berrimah Veterinary Laboratories (NT) for phylogenetics and whole genome sequencing studies done in this Project.

**Dr Kitman Dyrting** and technical staff from Berrimah Veterinary Laboratories (NT) assisted with collaborating on the Inter-laboratory PCR validation studies.

**Dr Brian Jones and technical staff of Western Australian Fisheries** assisted with collaborating on the Inter-laboratory PCR validation.

**Dr David Blyde** & **Marnie Horton** of *Sea World,* provided unidentified bacterial strains from sick stingrays to the PI at TAAHL for further bacterial identification and confirmation as *S. agalactiae*, thus contributing to scientific components of this Project.

**The following staff of the DAFF Northern Fisheries Centre and DAFF Fisheries, Cairns** are thanked for their valuable in-kind contribution on the project;

**Mr Adam Reynolds** and **Richard Knuckey** provided a depth of expertise, providing juvenile grouper fish for the running of the four experimental challenge trials, from multiple grouper spawnings done at NFC. This was not an easy task. Adam and Richard worked tirelessly around the clock to successfully spawn wild-caught Queensland grouper broodstock in captivity, rear the larvae, wean fish onto commercial diets and onto healthy surviving juveniles that could then be used for this project, under a DAFF Animal Ethics Permit.

**Mr Malcolm Pearce** provided much appreciated knowledge and discussions on tilapia and is thanked for his contribution and assistance with providing staff and electro-fishing equipment for tilapia sampling, for surveillance studies. Malcolm provided good discussions on tilapia and feral pest fishes, and is thanked for his expertise and knowledge on feral fish species in the North Queensland region.

**Mr John Russell** assisted with fish identification skills, of all wild caught fish species to species level, caught for the purposes of the fish surveillance studies, and is greatly thanked for his time, expertise and skills in fish identification.

**Mr Julian O'Brien, Mr Geoff McPherson**, **Mr Stuart Hyland**, and **Mr Chad Lunow** are thanked for assisting with provision of samples from post mortems done on some dead grouper from the Cairns region. They also provided samples from some wild caught reef fish for the surveillance studies.

**Recreational & commercial fishermen of North Queensland,** including **Matt Vickers**, are greatly thanked for their valued time and assistance with sampling of wild fish and crab species in Trinity Inlet, Cairns, Weipa, Port Douglas, Townsville, Bowen, Ayr, Proserpine, Gladstone & Rockhampton, for the purposes of surveillance of wild fish and crustaceans for *S. agalactiae* for the targeted surveillance studies.

**The following groups and people contributed to this project by reporting dead or sick grouper to DAFF (from which bacterial isolates were obtained for the purposes for this research project)**

**DAFF Queensland Boating & Fisheries Patrol Officers** from Port Douglas, Cairns, Weipa, Townsville, Bowen, Mackay, Proserpine, Airlie beach, Rockhampton, Gladstone and Brisbane greatly assisted by reporting and retrieving dead grouper carcasses, some found sick or dying or dead and floating at sea, for the purposes of post mortem and bacterial isolation.

**The Australian Institute of Marine Science staff** reported several dead and sick grouper from the Cape Cleveland area to the PI for post mortem.

**Weipa commercial fishermen** & numerous members of the public from North Queensland reported many dead of dying Queensland grouper to the PI.

**Dr Col Limpus** of the **Department of Environment and Heritage** reported a dead grouper from the Bundaberg region.

**Mr Vern Veitch, Deputy councillor of Townsville City Council** provided valuable local support in the Townsville region for this project and also provided information and discussion on tilapia.

**Mr Viv Sinnamon** (Manager), and staff of the **Kowanyama Aboriginal Land and Natural Resources Management Office**, and members of the **Kowanyama Aboriginal community** in the Gulf of Carpentaria, are thanked for their valuable input and for providing information and reports on wild fish kills, sightings of dead or dying Queensland grouper, and of Queensland grouper skulls found washed up on beaches in the Gulf of Carpentaria and Northern Gulf region.

**Dr Mark Reid, Dr Adam Smith,** and **Dr Kirstin Dobbs**, of **GBRMPA (Great Barrier Reef Marine Park Authority** contributed to and supported this project in many ways. The integrated, multi-agency approach including the formation of a Queensland grouper technical working group in 2009, was much appreciated. A Fact Sheet was created in 2009 by GBRMPA and the PI, for assisting the public, commercial and recreational fishers in reporting dead grouper to the PI, just prior to this Project pre-proposal development (Appendix 12). GBRMPA staff co-ordinated and hosted a multi-organisational *Risk Assessment* (Appendix 10), in regards to identifying the potential risks, impacts and hazards of the bacterium *Streptococcus agalactiae* to wild fisheries and fauna within the Great Barrier Reef Marine Park.

**Note: The DAFF Biosecurity Queensland, Tropical & Aquatic Animal Health Laboratory (TAAHL) was closed in June 2012, during the duration of work for this project. This resulted in loss of the project technician appointed to the project, loss of all DAFF staff providing In-kind contributions to this project (apart from the project leader), and loss of laboratory resources.**

Finally a big big thank you to my wonderful family who were fully supportive during the entirety of this Project.

# APPENDIX 3. INJECTION CHALLENGE TRIAL (REPLICATE 1).

Summary of all laboratory test results for individual juvenile *E. lanceolatus* tested by PCR, bacteriology and histopathology analysis from all six treatment groups.

| Replicate & Treatment Group (dose bacteria injected cfu/ml) | Fish No | Fish Clinical signs | Fate of Fish | sample no. | Organ tested | TAAHL PCR result | TAAHL PCR whole fish Result (pooled) | No. Postive Fish/ total tested | UQ Bacteriology result | Pooled UQ Bacto results | Histology GG stain (organs) | Histology (whole fish) diagnosis | No Histo Positive fish/total tested | histology summary and comments |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| R1 T6 Injection ($10^8$ cfu/ml) | 1 | Congested anus, bilateral exophthalmos | ME | 1 | Kidney | + | Pos | 7/7 +ve | + | Pos | + | Pos | 7/7 +ve | Bacterial septicaemia.Gram Glynn-positive bacteria in all organs.Ophthalmitis, branchitis, pericarditis, splentiis, nephritis, hepatitis, enteritis, peritonitis, myositis, dermatitis. |
| | | | | 2 | Spleen | + | | | + | | + | | | |
| | | | | 3 | Brain | + | | | + | | + | | | |
| | 2 | Congested anus | ME | 4 | Kidney | + | Pos | | | | + | Pos | | Bacterial septicaemia.Gram Glynn-positive bacteria in all organs.Ophthalmitis, branchitis, pericarditis, splentiis, nephritis, hepatitis, enteritis, peritonitis, myositis, dermatitis. |
| | | | | 5 | Spleen | + | | | | | + | | | |
| | | | | 6 | Brain | + | | | | | + | | | |
| | 3 | Congested anus | ME | 7 | Kidney | + | Pos | | | | + | Pos | | Bacterial septicaemia.Gram Glynn-positive bacteria in all organs.Ophthalmitis, branchitis, pericarditis, splentiis, nephritis, hepatitis, enteritis, peritonitis, myositis, dermatitis. |
| | | | | 8 | Spleen | + | | | | | + | | | |
| | | | | 9 | Brain | + | | | | | + | | | |
| | 4 | Congested anus, bilateral exophthalmos | ME | 10 | Kidney | + | Pos | | + | Pos | + | Pos | | Bacterial septicaemia.Gram Glynn-positive bacteria in all organs.Ophthalmitis, branchitis, pericarditis, splentiis, nephritis, hepatitis, enteritis, peritonitis, myositis, dermatitis. |
| | | | | 11 | Spleen | + | | | + | | + | | | |
| | | | | 12 | Brain | + | | | + | | + | | | |
| | 5 | Congested anus, gills petechieal haemorrhages | D | 13 | Kidney | + | Pos | | | | + | Pos | | Bacterial septicaemia.Gram Glynn-positive bacteria in all organs.Ophthalmitis, branchitis, pericarditis, splentiis, nephritis, hepatitis, enteritis, peritonitis, myositis, dermatitis. |
| | | | | 14 | Spleen | + | | | | | + | | | |
| | | | | 15 | Brain | + | | | | | + | | | |
| | 6 | Congested anus, gills petechial haemorrhages | D | 16 | Kidney | + | Pos | | | | + | Pos | | Bacterial septicaemia.Gram Glynn-positive bacteria in all organs.Ophthalmitis, branchitis, pericarditis, splentiis, nephritis, hepatitis, enteritis, peritonitis, myositis, dermatitis. |
| | | | | 17 | Spleen | + | | | | | + | | | |
| | | | | 18 | Brain | + | | | | | + | | | |
| | 7 | Congested anus, bilateral exophthalmos | ME | 19 | Kidney | + | Pos | | | | + | Pos | | Bacterial septicaemia.Gram Glynn-positive bacteria in all organs.Ophthalmitis, branchitis, pericarditis, splentiis, nephritis, hepatitis, enteritis, peritonitis, myositis, dermatitis. |
| | | | | 20 | Spleen | + | | | | | + | | | |
| | | | | 21 | Brain | + | | | | | + | | | |
| R1 T5 Injection ($10^7$ cfu/ml) | 1 | Bilat exophthalmos, gill petechial haemorrhage | D | 1 | Kidney | + | Pos | 6/6 +ve | | | + | Pos | 6/6 +ve | Bacterial septicaemia.Gram Glynn-positive bacteria in all organs.Ophthalmitis, branchitis, pericarditis, splentiis, nephritis, hepatitis, enteritis, peritonitis, myositis, dermatitis. |
| | | | | 2 | Spleen | + | | | | | + | | | |
| | | | | 3 | Brain | + | | | | | + | | | |
| | 2 | Congested anus | D | 4 | Kidney | + | Pos | | + | Pos | + | Pos | | Bacterial septicaemia.Gram Glynn-positive bacteria in all organs.Ophthalmitis, branchitis, pericarditis, splentiis, nephritis, hepatitis, enteritis, peritonitis, myositis, dermatitis. |
| | | | | 5 | Spleen | + | | | + | | + | | | |
| | | | | 6 | Brain | + | | | + | | + | | | |
| | 3 | Congested anus, bilateral exophthalmos | ME | 7 | Kidney | + | Pos | | | | + | Pos | | Bacterial septicaemia.Gram Glynn-positive bacteria in all organs.Ophthalmitis, branchitis, pericarditis, splentiis, nephritis, hepatitis, enteritis, peritonitis, myositis, dermatitis. |
| | | | | 8 | Spleen | + | | | | | + | | | |
| | | | | 9 | Brain | + | | | | | + | | | |
| | 4 | Congested anus, bilateral exophthalmos | D | 10 | Kidney | + | Pos | | | | + | Pos | | Bacterial septicaemia.Gram Glynn-positive bacteria in all organs.Ophthalmitis, branchitis, pericarditis, splentiis, nephritis, hepatitis, enteritis, peritonitis, myositis, dermatitis. |
| | | | | 11 | Spleen | + | | | | | + | | | |
| | | | | 12 | Brain | + | | | | | + | | | |
| | 5 | Congested anus, bilateral exophthalmos, gill haemor. | ME | 13 | Kidney | + | Pos | | | | + | Pos | | Bacterial septicaemia.Gram Glynn-positive bacteria in all organs.Ophthalmitis, branchitis, pericarditis, splentiis, nephritis, hepatitis, enteritis, peritonitis, myositis, dermatitis. |
| | | | | 14 | Spleen | + | | | | | + | | | |
| | | | | 15 | Brain | + | | | | | + | | | |
| | 6 | N/O | ME | 16 | Kidney | + | Pos | | N | Pos | + | Pos | | Bacterial septicaemia.Gram Glynn-positive bacteria in all organs.Ophthalmitis, branchitis, pericarditis, splentiis, nephritis, hepatitis, enteritis, peritonitis, myositis, dermatitis. |
| | | | | 17 | Spleen | + | | | + | | + | | | |
| | | | | 18 | Brain | + | | | + | | + | | | |
| R1 T4 Injection ($10^6$ cfu/ml) | 1 | Gills with petechial haemorrhages | D | 1 | Kidney | + | Pos | 5/5 +ve | + | Pos | + | Pos | 5/5 +ve | Bacterial septicaemia.Gram Glynn-positive bacteria in all organs.Ophthalmitis, branchitis, pericarditis, splentiis, nephritis, hepatitis, enteritis, peritonitis, myositis, dermatitis. |
| | | | | 2 | Spleen | + | | | + | | + | | | |
| | | | | 3 | Brain | + | | | N | | + | | | |
| | 2 | Gills with petechial haemorrhages | D | 4 | Kidney | + | Pos | | | | + | Pos | | Bacterial septicaemia.Gram Glynn-positive bacteria in all organs.Ophthalmitis, branchitis, pericarditis, splentiis, nephritis, hepatitis, enteritis, peritonitis, myositis, dermatitis. |
| | | | | 5 | Spleen | + | | | | | + | | | |
| | | | | 6 | Brain | missing | | | | | + | | | |
| | 3 | upside-down, bilateral exophthalmos | ME | 7 | Kidney | + | Pos | | | | + | Pos | | Bacterial septicaemia.Gram Glynn-positive bacteria in all organs.Ophthalmitis, branchitis, pericarditis, splentiis, nephritis, hepatitis, enteritis, peritonitis, myositis, dermatitis. |
| | | | | 8 | Spleen | + | | | | | + | | | |
| | | | | 9 | Brain | missing | | | | | + | | | |
| | 4 | Congested anus, bilateral exophthalmos | D | 10 | Kidney | + | Pos | | N | Pos | + | Pos | | Bacterial septicaemia.Gram Glynn-positive bacteria in all organs.Ophthalmitis, branchitis, pericarditis, splentiis, nephritis, hepatitis, enteritis, peritonitis, myositis, dermatitis. |
| | | | | 11 | Spleen | + | | | + | | + | | | |
| | | | | 12 | Brain | + | | | + | | + | | | |
| | 5 | Congested anus, bilateral exophthalmos | ME | 13 | Kidney | + | Pos | | | | + | Pos | | Bacterial septicaemia.Gram Glynn-positive bacteria in all organs.Ophthalmitis, branchitis, pericarditis, splentiis, nephritis, hepatitis, enteritis, peritonitis, myositis, dermatitis. |
| | | | | 14 | Spleen | + | | | | | + | | | |
| | | | | 15 | Brain | + | | | | | + | | | |
| R1 T3 Injection ($10^5$ cfu/ml) | 1 | Found dead, no external signs | D | 1 | Kidney | + | Pos | 5/5 +ve | + | Pos | + | Pos | 5/5 +ve | Bacterial septicaemia.Gram Glynn-positive bacteria in all organs.Ophthalmitis, branchitis, pericarditis, splentiis, nephritis, hepatitis, enteritis, peritonitis, myositis, dermatitis. |
| | | | | 2 | Spleen | + | | | + | | + | | | |
| | | | | 3 | Brain | + | | | N | | + | | | |
| | 2 | Congested anus | D | 4 | Kidney | + | Pos | | | | + | Pos | | Bacterial septicaemia.Gram Glynn-positive bacteria in all organs.Ophthalmitis, branchitis, pericarditis, splentiis, nephritis, hepatitis, enteritis, peritonitis, myositis, dermatitis. |
| | | | | 5 | Spleen | + | | | | | + | | | |
| | | | | 6 | Brain | + | | | | | + | | | |
| | 3 | Congested anus, bilateral exophthalmos | ME | 7 | Kidney | + | Pos | | | | + | Pos | | Bacterial septicaemia.Gram Glynn-positive bacteria in all organs.Ophthalmitis, branchitis, pericarditis, splentiis, nephritis, hepatitis, enteritis, peritonitis, myositis, dermatitis. |
| | | | | 8 | Spleen | + | | | | | + | | | |
| | | | | 9 | Brain | + | | | | | + | | | |
| | 4 | Congested anus, bilat exophth, gill petechial haemorrgahes | D | 10 | Kidney | + | Pos | | + | Pos | + | Pos | | Bacterial septicaemia.Gram Glynn-positive bacteria in all organs.Ophthalmitis, branchitis, pericarditis, splentiis, nephritis, hepatitis, enteritis, peritonitis, myositis, dermatitis. |
| | | | | 11 | Spleen | + | | | + | | + | | | |
| | | | | 12 | Brain | + | | | + | | + | | | |
| | 5 | Congested anus, bilateral exophthalmos | D | 13 | Kidney | + | Pos | | | | + | Pos | | Bacterial septicaemia.Gram Glynn-positive bacteria in all organs.Ophthalmitis, branchitis, pericarditis, splentiis, nephritis, hepatitis, enteritis, peritonitis, myositis, dermatitis. |
| | | | | 14 | Spleen | + | | | | | + | | | |
| | | | | 15 | Brain | + | | | | | + | | | |
| R1 T2 Injection ($10^4$ cfu/ml) | 1 | congested anus, splenomegaly | D | 1 | Kidney | + | Pos | 5/5 +ve | + | Pos | + | Pos | 5/5 +ve | Bacterial septicaemia.Gram Glynn-positive bacteria in all organs.Ophthalmitis, branchitis, pericarditis, splentiis, nephritis, hepatitis, enteritis, peritonitis, myositis, dermatitis. |
| | | | | 2 | Spleen | + | | | + | | + | | | |
| | | | | 3 | Brain | + | | | N | | + | | | |
| | 2 | Congested anus, bilateral exophthalmos | ME | 4 | Kidney | + | Pos | | N | Pos | + | Pos | | Bacterial septicaemia.Gram Glynn-positive bacteria in all organs.Ophthalmitis, branchitis, pericarditis, splentiis, nephritis, hepatitis, enteritis, peritonitis, myositis, dermatitis. |
| | | | | 5 | Spleen | + | | | + | | + | | | |
| | | | | 6 | Brain | + | | | + | | + | | | |
| | 3 | Congested anus, bilateral exophthalmos | ME | 7 | Kidney | + | Pos | | | | + | Pos | | Bacterial septicaemia.Gram Glynn-positive bacteria in all organs.Ophthalmitis, branchitis, pericarditis, splentiis, nephritis, hepatitis, enteritis, peritonitis, myositis, dermatitis. |
| | | | | 8 | Spleen | + | | | | | + | | | |
| | | | | 9 | Brain | + | | | | | + | | | |
| | 4 | upside-down,congested anus, bilateral exophthalmos | ME | 10 | Kidney | + | Pos | | | | + | Pos | | Bacterial septicaemia.Gram Glynn-positive bacteria in all organs.Ophthalmitis, branchitis, pericarditis, splentiis, nephritis, hepatitis, enteritis, peritonitis, myositis, dermatitis. |
| | | | | 11 | Spleen | + | | | | | + | | | |
| | | | | 12 | Brain | + | | | | | + | | | |
| | 5 | Erratic swimming congested anus, bilateral exophthalmos | ME | 13 | Kidney | + | Pos | | | | + | Pos | | Bacterial septicaemia.Gram Glynn-positive bacteria in all organs.Ophthalmitis, branchitis, pericarditis, splentiis, nephritis, hepatitis, enteritis, peritonitis, myositis, dermatitis. |
| | | | | 14 | Spleen | + | | | | | + | | | |
| | | | | 15 | Brain | + | | | | | + | | | |
| R1 T1 Injection ($10^3$ cfu/ml) | 1 | Congested anus | ME | 1 | Kidney | N | Pos | 6/6+ve | | | + | Pos | 6/6+ve | Bacterial septicaemia.Gram Glynn-positive bacteria in all organs.Ophthalmitis, branchitis, pericarditis, splentiis, nephritis, hepatitis, enteritis, peritonitis, myositis, dermatitis. |
| | | | | 2 | Spleen | + | | | | | + | | | |
| | | | | 3 | Brain | + | | | | | + | | | |
| | 2 | Congested anus, bilat exophthalmos | ME | 4 | Kidney | + | Pos | | + | Pos | + | Pos | | Bacterial septicaemia.Gram Glynn-positive bacteria in all organs.Ophthalmitis, branchitis, pericarditis, splentiis, nephritis, hepatitis, enteritis, peritonitis, myositis, dermatitis. |
| | | | | 5 | Spleen | + | | | + | | + | | | |
| | | | | 6 | Brain | + | | | + | | + | | | |
| | 3 | Congested anus, bilat exophthalmos, ascites | ME | 7 | Kidney | + | Pos | | + | Pos | + | Pos | | Bacterial septicaemia.Gram Glynn-positive bacteria in all organs.Ophthalmitis, branchitis, pericarditis, splentiis, nephritis, hepatitis, enteritis, peritonitis, myositis, dermatitis. |
| | | | | 8 | Spleen | + | | | + | | + | | | |
| | | | | 9 | Brain | + | | | + | | + | | | |
| | 4 | Congested anus, bilat exophthalmos, ascites | D | 10 | Kidney | + | Pos | | | | + | Pos | | Bacterial septicaemia.Gram Glynn-positive bacteria in all organs.Ophthalmitis, branchitis, pericarditis, splentiis, nephritis, hepatitis, enteritis, peritonitis, myositis, dermatitis. |
| | | | | 11 | Spleen | + | | | | | + | | | |
| | | | | 12 | Brain | + | | | | | + | | | |
| | 5 | Congested anus, | D | 13 | Kidney | + | Pos | | | | + | Pos | | Bacterial septicaemia.Gram Glynn-positive bacteria in all organs.Ophthalmitis, branchitis, pericarditis, splentiis, nephritis, hepatitis, enteritis, peritonitis, myositis, dermatitis. |
| | | | | 14 | Spleen | + | | | | | + | | | |
| | | | | 15 | Brain | + | | | | | + | | | |
| | 6 | N/O | D | 16 | Kidney | + | Pos | | | | + | Pos | | Bacterial septicaemia.Gram Glynn-positive bacteria in all organs.Ophthalmitis, branchitis, pericarditis, splentiis, nephritis, hepatitis, enteritis, peritonitis, myositis, dermatitis. |
| | | | | 17 | Spleen | + | | | | | + | | | |
| | | | | 18 | Brain | + | | | | | + | | | |

# APPENDIX 4. INJECTION CHALLENGE TRIAL (REPLICATE 2).

Summary of all laboratory test results, for individual juvenile *E. lanceolatus* tested by PCR, bacteriology and histopathology analysis.

| Replicate & Treatment Dose (bacteria cfu/ml) | Fish No. | Fish Clinical signs | Fate of Fish | Fish sample no. | organ tested | TAAHL PCR result | Whole Fish (pooled) TAAHL PCR Result | No. Postive Fish/total fish tested | UQ Bacto (indiv. organ) result | UQ Bacto (pooled result) | Histology Gram Glynn stain (organs) | Histology (whole fish) diagnosis | No. Histo Positive fish/No tested | Histopathology summary |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| R2 T6 Injection ($10^8$ cfu/ml) | 1 | no gross pathology observed | D | 1 | Kidney | + | Pos | 7/7 +ve | + | Pos | + | Pos | 7/7 +ve | Bacterial septicaemia.Gram Glynn-positive bacteria in all organs.Ophthalmitis, branchitis, pericarditis, splentiis, nephritis, hepatitis, enteritis, peritonitis, myositis, dermatitis. |
| | | | | 2 | Spleen | + | | | N | | + | | | |
| | | | | 3 | missing | missing | | | + | | + | | | |
| | 2 | congested aus | D | 4 | Kidney | + | Pos | | + | Pos | + | Pos | | Bacterial septicaemia.Gram Glynn-positive bacteria in all organs.Ophthalmitis, branchitis, pericarditis, splentiis, nephritis, hepatitis, enteritis, peritonitis, myositis, dermatitis. |
| | | | | 5 | Spleen | + | | | + | | + | | | |
| | | | | 6 | Brain | + | | | + | | + | | | |
| | 3 | N/O | ME | 7 | Kidney | + | Pos | | | | + | Pos | | Bacterial septicaemia.Gram Glynn-positive bacteria in all organs.Ophthalmitis, branchitis, pericarditis, splentiis, nephritis, hepatitis, enteritis, peritonitis, myositis, dermatitis. |
| | | | | 8 | Spleen | + | | | | | + | | | |
| | | | | 9 | Brain | + | | | | | + | | | |
| | 4 | N/O | ME | 10 | Kidney | + | Pos | | | | + | Pos | | Bacterial septicaemia.Gram Glynn-positive bacteria in all organs.Ophthalmitis, branchitis, pericarditis, splentiis, nephritis, hepatitis, enteritis, peritonitis, myositis, dermatitis. |
| | | | | 11 | Spleen | + | | | | | + | | | |
| | | | | 12 | Brain | + | | | | | + | | | |
| | 5 | Congested anus, bilateral exophthalmos | D | 13 | Kidney | + | Pos | | | | + | Pos | | Bacterial septicaemia.Gram Glynn-positive bacteria in all organs.Ophthalmitis, branchitis, pericarditis, splentiis, nephritis, hepatitis, enteritis, peritonitis, myositis, dermatitis. |
| | | | | 14 | Spleen | + | | | | | + | | | |
| | | | | 15 | Brain | + | | | | | + | | | |
| | 6 | Congested anus, bilat exoph, gill haemorrhage | D | 16 | Kidney | + | Pos | | | | + | Pos | | Bacterial septicaemia.Gram Glynn-positive bacteria in all organs.Ophthalmitis, branchitis, pericarditis, splentiis, nephritis, hepatitis, enteritis, peritonitis, myositis, dermatitis. |
| | | | | 17 | Spleen | + | | | | | + | | | |
| | | | | 18 | Brain | + | | | | | + | | | |
| | 7 | congested anus | D | 19 | Kidney | + | Pos | | | | + | Pos | | Bacterial septicaemia.Gram Glynn-positive bacteria in all organs.Ophthalmitis, branchitis, pericarditis, splentiis, nephritis, hepatitis, enteritis, peritonitis, myositis, dermatitis. |
| | | | | 20 | Spleen | + | | | | | + | | | |
| | | | | 21 | Brain | + | | | | | + | | | |
| R2 T5 Injection ($10^7$ cfu/ml) | 1 | Congested anus, bilateral exophthalmos | D | 1 | Kidney | + | Pos | 6/6 +ve | + | Pos | + | Pos | 6/6 +ve | Bacterial septicaemia.Gram Glynn-positive bacteria in all organs.Ophthalmitis, branchitis, pericarditis, splentiis, nephritis, hepatitis, enteritis, peritonitis, myositis, dermatitis. |
| | | | | 2 | Spleen | + | | | + | | + | | | |
| | | | | 3 | Brain | + | | | + | | + | | | |
| | 2 | Congested anus, bilateral exophthalmos | D | 4 | Kidney | + | Pos | | + | Pos | + | Pos | | Bacterial septicaemia.Gram Glynn-positive bacteria in all organs.Ophthalmitis, branchitis, pericarditis, splentiis, nephritis, hepatitis, enteritis, peritonitis, myositis, dermatitis. |
| | | | | 5 | Spleen | + | | | + | | + | | | |
| | | | | 6 | Brain | + | | | + | | + | | | |
| | 3 | Congested anus, bilateral exophthalmos | ME | 7 | Kidney | + | Pos | | | | + | Pos | | Bacterial septicaemia.Gram Glynn-positive bacteria in all organs.Ophthalmitis, branchitis, pericarditis, splentiis, nephritis, hepatitis, enteritis, peritonitis, myositis, dermatitis. |
| | | | | 8 | Spleen | + | | | | | + | | | |
| | | | | 9 | Brain | + | | | | | + | | | |
| | 4 | Congested anus, bilateral exophthalmos | ME | 10 | Kidney | + | Pos | | | | + | Pos | | Bacterial septicaemia.Gram Glynn-positive bacteria in all organs.Ophthalmitis, branchitis, pericarditis, splentiis, nephritis, hepatitis, enteritis, peritonitis, myositis, dermatitis. |
| | | | | 11 | Spleen | + | | | | | + | | | |
| | | | | 12 | Brain | + | | | | | + | | | |
| | 5 | N/O | ME | 13 | Kidney | + | Pos | | | | + | Pos | | Bacterial septicaemia.Gram Glynn-positive bacteria in all organs.Ophthalmitis, branchitis, pericarditis, splentiis, nephritis, hepatitis, enteritis, peritonitis, myositis, dermatitis. |
| | | | | 14 | Spleen | + | | | | | + | | | |
| | | | | 15 | Brain | + | | | | | + | | | |
| | 6 | Congested anus, bilateral exophthalmos | D | 16 | Kidney | + | Pos | | | | + | Pos | | Bacterial septicaemia.Gram Glynn-positive bacteria in all organs.Ophthalmitis, branchitis, pericarditis, splentiis, nephritis, hepatitis, enteritis, peritonitis, myositis, dermatitis. |
| | | | | 17 | Spleen | + | | | | | + | | | |
| | | | | 18 | Brain | + | | | | | + | | | |
| R2 T4 Injection ($10^6$ cfu/ml) | 1 | Congested anus, bilateral exophthalmos | ME | 1 | Kidney | + | Pos | 5/5 +ve | + | Pos | + | Pos | 5/5 +ve | Bacterial septicaemia.Gram Glynn-positive bacteria in all organs.Ophthalmitis, branchitis, pericarditis, splentiis, nephritis, hepatitis, enteritis, peritonitis, myositis, dermatitis. |
| | | | | 2 | Spleen | + | | | + | | + | | | |
| | | | | 3 | Brain | + | | | N | | + | | | |
| | 2 | Congested anus | D | 4 | Kidney | + | Pos | | + | Pos | + | Pos | | Bacterial septicaemia.Gram Glynn-positive bacteria in all organs.Ophthalmitis, branchitis, pericarditis, splentiis, nephritis, hepatitis, enteritis, peritonitis, myositis, dermatitis. |
| | | | | 5 | Spleen | + | | | + | | + | | | |
| | | | | 6 | Brain | + | | | + | | + | | | |
| | 3 | Congested anus, bilateral exophthalmos | ME | 7 | Kidney | + | Pos | | | | + | Pos | | Bacterial septicaemia.Gram Glynn-positive bacteria in all organs.Ophthalmitis, branchitis, pericarditis, splentiis, nephritis, hepatitis, enteritis, peritonitis, myositis, dermatitis. |
| | | | | 8 | Spleen | + | | | | | + | | | |
| | | | | 9 | Brain | + | | | | | + | | | |
| | 4 | Congested anus, bilateral exophthalmos | D | 10 | Kidney | + | Pos | | | | + | Pos | | Bacterial septicaemia.Gram Glynn-positive bacteria in all organs.Ophthalmitis, branchitis, pericarditis, splentiis, nephritis, hepatitis, enteritis, peritonitis, myositis, dermatitis. |
| | | | | 11 | Spleen | + | | | | | + | | | |
| | | | | 12 | Brain | + | | | | | + | | | |
| | 5 | Congested anus, bilateral exophthalmos | ME | 13 | Kidney | + | Pos | | | | + | Pos | | Bacterial septicaemia.Gram Glynn-positive bacteria in all organs.Ophthalmitis, branchitis, pericarditis, splentiis, nephritis, hepatitis, enteritis, peritonitis, myositis, dermatitis. |
| | | | | 14 | Spleen | + | | | | | + | | | |
| | | | | 15 | Brain | + | | | | | + | | | |
| R2 T3 Injection ($10^5$ cfu/ml) | 1 | Congested anus | D | 1 | Kidney | + | Pos | 5/5 +ve | + | Pos | + | Pos | 5/5 +ve | Bacterial septicaemia.Gram Glynn-positive bacteria in all organs.Ophthalmitis, branchitis, pericarditis, splentiis, nephritis, hepatitis, enteritis, peritonitis, myositis, dermatitis. |
| | | | | 2 | Spleen | + | | | + | | + | | | |
| | | | | 3 | Brain | + | | | + | | + | | | |
| | 2 | Congested anus, bilateral exophthalmos | D | 4 | Kidney | + | Pos | | + | Pos | + | Pos | | Bacterial septicaemia.Gram Glynn-positive bacteria in all organs.Ophthalmitis, branchitis, pericarditis, splentiis, nephritis, hepatitis, enteritis, peritonitis, myositis, dermatitis. |
| | | | | 5 | Spleen | + | | | + | | + | | | |
| | | | | 6 | Brain | + | | | + | | + | | | |
| | 3 | Congested anus, bilateral exophthalmos | D | 7 | Kidney | + | Pos | | | | + | Pos | | Bacterial septicaemia.Gram Glynn-positive bacteria in all organs.Ophthalmitis, branchitis, pericarditis, splentiis, nephritis, hepatitis, enteritis, peritonitis, myositis, dermatitis. |
| | | | | 8 | Spleen | + | | | | | + | | | |
| | | | | 9 | Brain | + | | | | | + | | | |
| | 4 | N/O | D | 10 | Kidney | + | Pos | | | | + | Pos | | Bacterial septicaemia.Gram Glynn-positive bacteria in all organs.Ophthalmitis, branchitis, pericarditis, splentiis, nephritis, hepatitis, enteritis, peritonitis, myositis, dermatitis. |
| | | | | 11 | Spleen | + | | | | | + | | | |
| | | | | 12 | Brain | + | | | | | + | | | |
| | 5 | Congested anus | ME | 13 | Kidney | + | Pos | | | | + | Pos | | Bacterial septicaemia.Gram Glynn-positive bacteria in all organs.Ophthalmitis, branchitis, pericarditis, splentiis, nephritis, hepatitis, enteritis, peritonitis, myositis, dermatitis. |
| | | | | 14 | Spleen | + | | | | | + | | | |
| | | | | 15 | Brain | + | | | | | + | | | |
| R2 T2 Injection ($10^4$ cfu/ml) | 1 | Congested anus | D | 1 | Kidney | + | Pos | 4/4 +ve | + | Pos | + | Pos | 4/4 +ve | Bacterial septicaemia.Gram Glynn-positive bacteria in all organs.Ophthalmitis, branchitis, pericarditis, splentiis, nephritis, hepatitis, enteritis, peritonitis, myositis, dermatitis. |
| | | | | 2 | Spleen | + | | | + | | + | | | |
| | | | | 3 | Brain | + | | | + | | + | | | |
| | 2 | Congested anus | ME | 4 | Kidney | + | Pos | | + | Pos | + | Pos | | Bacterial septicaemia.Gram Glynn-positive bacteria in all organs.Ophthalmitis, branchitis, pericarditis, splentiis, nephritis, hepatitis, enteritis, peritonitis, myositis, dermatitis. |
| | | | | 5 | Spleen | + | | | + | | + | | | |
| | | | | 6 | Brain | + | | | + | | + | | | |
| | 3 | Congested anus, bilateral exophthalmos | ME | 7 | Kidney | + | Pos | | | | + | Pos | | Bacterial septicaemia.Gram Glynn-positive bacteria in all organs.Ophthalmitis, branchitis, pericarditis, splentiis, nephritis, hepatitis, enteritis, peritonitis, myositis, dermatitis. |
| | | | | 8 | Spleen | + | | | | | + | | | |
| | | | | 9 | Brain | + | | | | | + | | | |
| | 4 | Congested anus, bilateral exophthalmos | ME | 10 | Kidney | + | Pos | | | | + | Pos | | Bacterial septicaemia.Gram Glynn-positive bacteria in all organs.Ophthalmitis, branchitis, pericarditis, splentiis, nephritis, hepatitis, enteritis, peritonitis, myositis, dermatitis. |
| | | | | 11 | Spleen | + | | | | | + | | | |
| | | | | 12 | Brain | + | | | | | + | | | |
| R2 T1 Injection (103 cfu/ml) | 1 | Congested anus, bilateral exophthalmos | D | 1 | Kidney | + | Pos | 6/6 +ve | + | Pos | + | Pos | 5/5+ve | Bacterial septicaemia.Gram Glynn-positive bacteria in all organs.Ophthalmitis, branchitis, pericarditis, splentiis, nephritis, hepatitis, enteritis, peritonitis, myositis, dermatitis. |
| | | | | 2 | Spleen | + | | | + | | + | | | |
| | | | | 3 | Brain | + | | | + | | + | | | |
| | 2 | Congested anus, bilateral exophthalmos | ME | 4 | Kidney | + | Pos | | N | Pos | + | Pos | | Bacterial septicaemia.Gram Glynn-positive bacteria in all organs.Ophthalmitis, branchitis, pericarditis, splentiis, nephritis, hepatitis, enteritis, peritonitis, myositis, dermatitis. |
| | | | | 5 | Spleen | + | | | + | | + | | | |
| | | | | 6 | Brain | + | | | N | | + | | | |
| | 3 | Congested anus | ME | 7 | Kidney | + | Pos | | | | N/T | Pos | | Bacterial septicaemia.Gram Glynn-positive bacteria in all organs.Ophthalmitis, branchitis, pericarditis, splentiis, nephritis, hepatitis, enteritis, peritonitis, myositis, dermatitis. |
| | | | | 8 | Spleen | + | | | | | + | | | |
| | | | | 9 | Brain | + | | | | | + | | | |
| | 4 | Congested anus, bilateral exophthalmos | ME | 10 | Kidney | + | Pos | | | | + | Pos | | Bacterial septicaemia.Gram Glynn-positive bacteria in all organs.Ophthalmitis, branchitis, pericarditis, splentiis, nephritis, hepatitis, enteritis, peritonitis, myositis, dermatitis. |
| | | | | 11 | Spleen | + | | | | | + | | | |
| | | | | 12 | Brain | + | | | | | + | | | |
| | 5 | moribund, survivor, euthanased | MSE | 13 | Kidney | + | Pos | | | | N | Pos | | Gram Glynn-positive bacteria in brain, spleen, gills. Mild Iritis, mild pericarditis, iritis lots MMC in spleen, meningitis, mild branchitis. |
| | | | | 14 | Spleen | + | | | | | N | | | |
| | | | | 15 | Brain | + | | | | | + | | | |
| | 6 | survivor, euthanased | SE | 16 | Kidney | + | Pos | | | | + | Pos | | Gram Glynn-positive bacteria in brain, heart, head kidney. Mild choroiditis, mild pericarditis, lots MMC in spleen, mild branchitis. |
| | | | | 17 | Spleen | + | | | | | + | | | |
| | | | | 18 | Brain | + | | | | | + | | | |

D=Dead; ME=Moribund euthanased; SE=Survivor euthanased; N=Negative;+,+ve,Pos=Positive.N/O=not observed.

# APPENDIX 5. IMMERSION CHALLENGE TRIAL (REPLICATE 1).

Summary of all laboratory test results for individual juvenile *E. lanceolatus* tested by PCR, bacteriology and histopathology analysis. Fish were immersed for 1 hour in seawater inoculated with low, medium or high doses of *S. agalactiae*, respectively: $10^4$, $10^5$, and $10^6$ cfu $ml^{-1}$.

| Replicate & immersion Treatment (dose bacteria cfu /ml) | Fish No. | Fish Clinical signs | Fate of Fish | Fish sample no. | Organ tested | TAAHL PCR result | TAAHL PCR whole fish result (pooled) | No. PCR Positive Fish/total fish tested | UQ Bacto indiv. Organ Result | UQ (pooled) bacto result | TAAHL Histology GG stain (organs) | TAAHL Histology GG stain (Pooled organs result) | No GG Pos fish/No tested | Histopathology summary and comments | TAAHL Histology whole fish result | No. Histo Pos fish/No tested |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| R1 & R2 Negative Control | 1 | NCXS | SE | 1 | Kidney | N | N | 0/4 +ve | N | N | N | N | 0/4 +ve | negative | N | 0/4 +ve |
| | | | | 2 | Spleen | N | | | N | | N | | | | | |
| | | | | 3 | Brain | N | | | N | | N | | | | | |
| | 2 | NCXS | SE | 4 | Kidney | N | N | | N/T | N/T | N | N | | Fish had 3 large granulomas containing short rod-shaped bacteria in liver, mild pericarditis. Diagnosis: gram-negative bacterial infection confined to liver. | N | |
| | | | | 5 | Spleen | N | | | N/T | | N | | | | | |
| | | | | 6 | Brain | N | | | N/T | | N | | | | | |
| | 3 | NCXS | SE | 7 | Kidney | N | N | | N | N | N | N | | negative | N | |
| | | | | 8 | Spleen | N | | | N | | N | | | | | |
| | | | | 9 | Brain | N | | | N | | N | | | | | |
| | 4 | NCXS | SE | 10 | Kidney | N | N | | N/T | N/T | N | N | | negative | N | |
| | | | | 11 | Spleen | N | | | N/T | | N | | | | | |
| | | | | 12 | Brain | N | | | N/T | | N | | | | | |
| R1 & R2 Positive control (injected with 10^6 cfu/fish bacteria) | 1 | both eyes exophthalmia | EM | 1 | Kidney | + | Pos | 4/4 +ve | N/T | N/T | + | Pos | 4/4 +ve | GG +ve bacteria (in gill blood vessel lumen, gill lamellae) severe ophthalmitis, marked choroiditis, severe pericarditis, splenic congestion, branchitiis, enteritis, pacnreatitis | Pos | 4/4 +ve |
| | | | | 2 | Spleen | + | | | N/T | | + | | | | | |
| | | | | 3 | Brain | + | | | N/T | | + | | | | | |
| | 2 | both eyes exophthalmia | EM | 4 | Kidney | + | Pos | | + | Pos | + | Pos | | GG +ve bacteria (brain, spleen, pericardium, endothelium, liver, swim bladder, peritoneaum, head kidney). severe ophthalmitis, marked choroiditis, pericarditis, splenic congestion, hepatitis, nephritis, meningitis. | Pos | |
| | | | | 5 | Spleen | + | | | + | | + | | | | | |
| | | | | 6 | Brain | + | | | + | | + | | | | | |
| | 3 | died, gills with petechial haemorrhages | D | 7 | Kidney | + | Pos | | N/T | N/T | + | Pos | | GG +ve bacteria (eye, brain, spleen, pericardium, endothelium, liver, intestine, gills, peritoneum, head kidney). severe ophthalmitis, marked choroiditis, pericarditis, splenic congestion, hepatitis, nephritis, meningitis. | Pos | |
| | | | | 8 | Spleen | + | | | N/T | | + | | | | | |
| | | | | 9 | Brain | + | | | N/T | | + | | | | | |
| | 4 | both eyes exophthalmia, erratic swimming | EM | 10 | Kidney | + | Pos | | + | Pos | + | Pos | | GG +ve bacteria (brain, spleen, kidney, heart, liver, peritoneum). severe ophthalmitis, marked choroiditis, pericarditis, splenic congestion, hepatitis, nephritis, meningitis. | Pos | |
| | | | | 11 | Spleen | + | | | + | | + | | | | | |
| | | | | 12 | Brain | + | | | + | | + | | | | | |
| R1 High (10^6) | 1 | NCXS | SE | 1 | Kidney | + | Pos | 4/4 +ve | N/T | N/T | N | N | 1/4 +ve | No bacteria detected, but pathology changes: mild choroiditis, granulomatous inflammation,, pericarditis, MMC in spleen, mild lymphocytic hepatitis,v. mild meningitis | N | 2/4 +ve |
| | | | | 2 | Spleen | N | | | N/T | | N | | | | | |
| | | | | 3 | Brain | N | | | N/T | | N | | | | | |
| | 2 | NCXS | SE | 4 | Kidney | + | Pos | | N/T | N/T | N | Pos | | GG+ve bacteria (skin, muscle,choroid, spleen), marked dermatitis, splenitis, marked pericarditis of BA & V. | Pos | |
| | | | | 5 | Spleen | N | | | N/T | | + | | | | | |
| | | | | 6 | Brain | N | | | N/T | | N | | | | | |
| | 3 | red skin lesion | SE | 7 | Kidney | + | Pos | | N | N | N | N | | GG+ve Bacteria (in sloughed skin cells/ macrophages inbetween adjacent gill filaments) | N | |
| | | | | 8 | Spleen | N | | | N | | N | | | | | |
| | | | | 9 | Brain | + | | | N | | N | | | | | |
| | 4 | NCXS | SE | 10 | Kidney | + | Pos | | N/T | N/T | N | N | | GG+ve bacteria in macrophages in iris, dermatitis, mild lymphocytic hepatitis, mild pericarditis | Pos | |
| | | | | 11 | Spleen | N | | | N/T | | N | | | | | |
| | | | | 12 | Brain | N | | | N/T | | N | | | | | |
| R1 Medium (10^5) | 1 | NCXS | SE | 1 | Kidney | + | Pos | 1/4 +ve | N/T | N/T | N | N | 1/4 +ve | No bacteria detected, but some pathology; mild pericarditis, mild lymphocytic hepatitis, eyes normal | N | 1/4 +ve |
| | | | | 2 | Spleen | N | | | N/T | | N | | | | | |
| | | | | 3 | Brain | N | | | N/T | | N | | | | | |
| | 2 | NCXS | SE | 4 | Kidney | N | N | | N | N | N | N | | No bacteria, but some dermatitis of fin, mild pericarditis of V, BA, rare mononuclear inflammatory infiltrate at base gill filaments | N | |
| | | | | 5 | Spleen | N | | | N | | N | | | | | |
| | | | | 6 | Brain | N | | | N | | N | | | | | |
| | 3 | NCXS | SE | 7 | Kidney | N | N | | N/T | N/T | N | Pos | | GG +ve bacteria (meninges, splenic macrophages, HK macrophages), choroiditis, mild pericarditis, scattered necr HT cells in kidney, splenic congestion | Pos | |
| | | | | 8 | Spleen | N | | | N/T | | + | | | | | |
| | | | | 9 | Brain | N | | | N/T | | + | | | | | |
| | 4 | NCXS | SE | 10 | Kidney | N | N | | N | N | N | N | | negative | N | |
| | | | | 11 | Spleen | N | | | N | | N | | | | | |
| | | | | 12 | Brain | N | | | N | | N | | | | | |
| R1 Low (10^4) | 1 | NCXS | SE | 1 | Kidney | + | Pos | 3/4 +ve | N | N | N | N | 0/4 +ve | GG +ve bacteria (few only in eye choroid, in sloughed cells inbetween adjacent gill lamellae, in macrophages in peritoneal cavity, a few sloughed macrophages in mucosal epithelium) | Pos | 3/4 +ve |
| | | | | 2 | Spleen | + | | | N | | N | | | | | |
| | | | | 3 | Brain | N | | | N | | N | | | | | |
| | 2 | NCXS | SE | 4 | Kidney | N | Pos | | N/T | N/T | N | N | | GG +ve bacteria (in few macrophages only in heart ventricle, and in gills) | Pos | |
| | | | | 5 | Spleen | + | | | N/T | | N | | | | | |
| | | | | 6 | Brain | N | | | N/T | | N | | | | | |
| | 3 | NCXS | SE | 7 | Kidney | N | N | | N/T | N/T | N | N | | GG +ve bacteria (but only in sloughed macrophages inbetween adjacent gill lamellae) | N | |
| | | | | 8 | Spleen | N | | | N/T | | N | | | | | |
| | | | | 9 | Brain | N | | | N/T | | N | | | | | |
| | 4 | NCXS | SE | 10 | Kidney | N | Pos | | N | N | N | N | | GG +ve bacteria (in sloughed cells macrophages, inbetween adjacent gill lamellae, in macrophages in lamina propria | Pos | |
| | | | | 11 | Spleen | + | | | N | | N | | | | | |
| | | | | 12 | Brain | N | | | N | | N | | | | | |
| R1 Sentinel Control | 1 | NCXS | SE | 1 | Kidney | N | N | 0/4 +ve | N/T | N/T | N | N | 0/4 +ve | No bacteria detected. Some very mild pericarditis of ventricle, mild lymphocytic hepatitis, eyes normal | N | 0/4 +ve |
| | | | | 2 | Spleen | N | | | N/T | | N | | | | | |
| | | | | 3 | Brain | N | | | N/T | | N | | | | | |
| | 2 | NCXS | SE | 4 | Kidney | N | N | | N | N | N | N | | No bacteria detected.Very mild periarditis ventricle & BA, multifocal myocarditis, 1 x foci of endocarditis, mild lymphocytic hepatitis, eyes normal, fins; show dermaitis | N | |
| | | | | 5 | Spleen | N | | | N | | N | | | | | |
| | | | | 6 | Brain | N | | | N | | N | | | | | |
| | 3 | NCXS | SE | 7 | Kidney | N | N | | N/T | N/T | N | N | | No bacteria detected. Very mild pericarditis, (multifocal inflamm lesions of ventricle, BA), nephrosis, odd liver lesion, eyes normal. | N | |
| | | | | 8 | Spleen | N | | | N/T | | N | | | | | |
| | | | | 9 | Brain | N | | | N/T | | N | | | | | |
| | 4 | NCXS | SE | 10 | Kidney | N | N | | N/T | N/T | N | N | | No bacteria detected. Very mild pericarditis ( ventricle, BA), mild lymphocytic hepatitis, nephrosis, eyes normal. | N | |
| | | | | 11 | Spleen | N | | | N/T | | N | | | | | |
| | | | | 12 | Brain | N | | | N/T | | N | | | | | |

D=found dead; EM= moribund, euthanased; SE=survivor, euthanased; N=negative;+, Pos,+ve=positive; N/T=Not tested; NCXS=No Clinical Signs; R1=Replicate 1; R2=Replicate 2

# APPENDIX 6. IMMERSION CHALLENGE TRIAL (REPLICATE 2).

Summary of all laboratory test results for individual fish *E. lanceolatus* tested by PCR, bacteriology and histopathology analysis.

| Replicate & Immersion Treatment (dose bacteria cfu /ml) | Fish No. | Fish Clinical signs | Fate of Fish | Fish sample no. | Organ tested | TAAHL PCR result | TAAHL PCR Result (pooled organs) | No. PCR Pos Fish/total No. fish tested | UQ Bacto (individual organ) result | UQ Bacto Result (pooled) | TAAHL Histology Gram Glynn Result (organ) | TAAHL Histology Gram Glynn (pooled organs) | No.Gram Glynn Pos fish/No tested | TAAHL: Histopathology summary and comments | TAAHL Histo whole fish Result | No. Histology Pos Fish/No. tested |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| R2 High (10^6) | 1 | exophthalmos, erratic swimming, red skin lesin | D | 1 | Kidney | + | Pos | 3/4 +ve | + | Pos | + | Pos | 4/4 +ve | **GG+ve bacteria** (Br, Spl, H, Liv, Kid, eye, muscle),marked choroiditis, pericarditis, splenic congestion. | Pos | 4/4 +ve |
| | | | | 2 | Spleen | + | | | + | | + | | | | | |
| | | | | 3 | M | + | | | + | | + | | | | | |
| | 2 | exophthalmos, erratic swimming | D | 4 | Kidney | + | Pos | | N/T | N/T | + | Pos | | **GG+ve bacteria** (Brain, Spl, H, Liv, Kid, eye, muscle),marked choroiditis, pericarditis, splenic congestion . | Pos | |
| | | | | 5 | Spleen | + | | | N/T | | + | | | | | |
| | | | | 6 | Brain | + | | | N/T | | + | | | | | |
| | 3 | exophthalmos, erratic swimming | D | 7 | Kidney | + | Pos | | N/T | N/T | + | Pos | | **GG+ve bacteria** (Br, Spl, H, Liv, Kid, eye, muscle),marked choroiditis, ophthalmitis, pericarditis, splenic congestion, cellulitis, myositis, fascitiits. | Pos | |
| | | | | 8 | Spleen | + | | | N/T | | + | | | | | |
| | | | | 9 | Brain | + | | | N/T | | + | | | | | |
| | 4 | NCXS | SE | 10 | Kidney | N | N | | + | Pos | + | Pos | | **GG+ve bacteria** (Brain meninges, choroid, sloughed intestinal mucsal cells, muscle, gill blood vessels, kidney, peritoneal macrophages),marked choroiditis, pericarditis, meningitis, mysositis, vascular endothelial necrosis. | Pos | |
| | | | | 11 | Spleen | N | | | + | | + | | | | | |
| | | | | 12 | Brain | N | | | + | | + | | | | | |
| R2 Medium (10^5) | 1 | exophthalmos, erratic swimming | D | 1 | Kidney | + | Pos | 4/4 +ve | + | Pos | N | Pos | 3/4 +ve | **GG+ve bacteria** (choroid, skel muscle, brain, stomach, peritoneum ), marked choroiditis, pericarditis, splenic congestion | Pos | 3/4 +ve |
| | | | | 2 | Spleen | + | | | + | | N | | | | | |
| | | | | 3 | Brain | + | | | + | | + | | | | | |
| | 2 | exophthalmos, erratic swimming | D | 4 | Kidney | + | Pos | | + | Pos | + | Pos | | **GG+ve bacteria** (eye, spleen, Kid, heart, brain, stom, peritoneum, gills, liver, int ) marked choroiditis, pericarditis, splenic congestion. | Pos | |
| | | | | 5 | Spleen | + | | | + | | + | | | | | |
| | | | | 6 | Brain | + | | | + | | + | | | | | |
| | 3 | NCXS | M | 7 | Kidney | + | Pos | | N/T | N/T | N | N | | mild pathology showing some changes (splenic congestion, dermatitis, mild choroiditis). | N | |
| | | | | 8 | Spleen | + | | | N/T | | N | | | | | |
| | | | | 9 | Brain | + | | | N/T | | N | | | | | |
| | 4 | NCXS | M | 10 | Kidney | N | Pos | | N/T | N/T | N | Pos | | **GG +ve bacteria** (spleen lots of melanin, 1 single macrophage with bacteria inside), pathology showed some changes (spleen lots of melanin). | Pos | |
| | | | | 11 | Spleen | + | | | N/T | | + | | | | | |
| | | | | 12 | Brain | + | | | N/T | | N | | | | | |
| R2 Low (10^4) | 1 | NCXS | SE | 1 | Kidney | N | N | 3/4 +ve | N | N | N | N | 2/4 +ve | GG +ve bacteria (only seen in sloughed macrophages in between gill filaments) | N | 2/4 +ve |
| | | | | 2 | Spleen | N | | | N | | N | | | | | |
| | | | | 3 | Brain | N | | | N | | N | | | | | |
| | 2 | NCXS | SE | 4 | Kidney | + | Pos | | N | N | N | Pos | | **GG +ve bacteria** (inside choroid of eye and in connective tissue of skin). | Pos | |
| | | | | 5 | Spleen | + | | | N | | N | | | | | |
| | | | | 6 | Brain | + | | | N | | N | | | | | |
| | 3 | NCXS | SE | 7 | Kidney | N | Pos | | N/T | N/T | N | N | | **GG +ve bacteria** (only seen in sloughed macrophages in between gill filaments) | N | |
| | | | | 8 | Spleen | N | | | N/T | | N | | | | | |
| | | | | 9 | Brain | + | | | N/T | | N | | | | | |
| | 4 | NCXS | SE | 10 | Kidney | N | Pos | | N/T | N/T | N | Pos | | **GG +ve bacteria** (seen in connective tissue of eye and in sloughed macrophages inbetween gill filaments). | Pos | |
| | | | | 11 | Spleen | + | | | N/T | | N | | | | | |
| | | | | 12 | Brain | N | | | N/T | | N | | | | | |
| R2 sentinel Control | 1 | NCXS | SE | 1 | Kidney | N | Pos | 4/4 +ve | N | N | N | Pos | 2/4 +ve | **GG+ve bacteria** in peritoneal macrophages; mild choroiditis, mild perivsascular infl liver, peritonitis, mild AV valve endocarditis, pericarditis (ventricle0, intestinal oedema & inflam submucosa, steatitis. | Pos | 2/4 +ve |
| | | | | 2 | Spleen | N | | | N | | N | | | | | |
| | | | | 3 | Brain | + | | | N | | N | | | | | |
| | 2 | NCXS | SE | 4 | Kidney | + | Pos | | N/T | N/T | N | Pos | | **GG+ve bacteria** (third ventricle of brain), Mild choroiditis, marked dermaitits of fins, pericariditsi if ventricle, BA, myocarditis, mild hepatitis, mild meningitis, abnormal swim bladder | Pos | |
| | | | | 5 | Spleen | + | | | N/T | | N | | | | | |
| | | | | 6 | Brain | N | | | N/T | | + | | | | | |
| | 3 | NCXS | SE | 7 | Kidney | + | Pos | | N/T | N/T | N | N | | mild branchitis, mild iritis, mild pericarditis, splenic congestion, mild pancreatitis, hyeraemia submucosa. | N | |
| | | | | 8 | Spleen | + | | | N/T | | N | | | | | |
| | | | | 9 | Brain | + | | | N/T | | N | | | | | |
| | 4 | NCXS | SE | 10 | Kidney | N | Pos | | N/T | N/T | N | N | | mild choroiditis, mild pericarditis, mild pancreatitis, | N | |
| | | | | 11 | Spleen | N | | | N/T | | N | | | | | |
| | | | | 12 | Brain | + | | | N/T | | N | | | | | |

D=dead; SE=survivor, euthanased; M=moribund, euthanased; +,Pos,+ve=positive; N=negative; N/T=Not tested; NCXS=No Clinical Signs

# APPENDIX 7. ORAL CHALLENGE TRIAL (REPLICATES 1 & 2).

Summary of all laboratory test results for individual fish *E. lanceolatus* tested by PCR, bacteriology and histopathology analysis.

| Treatment Group | Fish No. | Fate of fish | Fish Clinical Signs observed | Fish Sample No. | organ tested | TAAHL PCR result | PCR (pooled) Fish Result | No. PCR pos fish/ total fish | UQ Bacto result | UQ Pooled Bacto result | Bacto Total No. Pos Fish | TAAHL Histology GG stain (organs) | TAAHL Histology GG stain (pooled organs) result | No.GG Pos fish/ No tested | Histopathology summary and comments | TAAHL Histology whole fish Result | No. Histo Pos fish/No. tested |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| R1 High Dose (10$^6$ cfu/fish) | 1 | EM | Immobile, ascites, odd heart shape, colour, Right eye exophthalmia | 1 | Kidney | + | P | 6/6+ve | + | P | 2/6 +ve | + | P | 1/6 +ve | **Marked pathology (as for Injected fish); GG positive bacteria in all organs & tissues.** | P | 1/6 +ve |
| | | | | 2 | Spleen | + | | | + | | | + | | | | | |
| | | | | 3 | Brain | + | | | + | | | + | | | | | |
| | 2 | E | Stomach full | 4 | Kidney | N | P | | N | N | | N | N | | Mild pathology: mild branchitis, iritis, hepatitis. No brain in sample sent. | N | |
| | | | | 5 | Spleen | + | | | N | | | N | | | | | |
| | | | | 6 | Brain | N | | | N | | | N/A | | | | | |
| | 3 | E | Stomach full, red caudal fin | 7 | Kidney | + | P | | + | P | | N | N | | Mild pathology: mild branchitis, iritis, hepatitis, mild nephritis. | N | |
| | | | | 8 | Spleen | + | | | N | | | N | | | | | |
| | | | | 9 | Brain | N | | | N | | | N | | | | | |
| | 4 | E | Stomach full, red caudal fin | 10 | Kidney | + | P | | N | N | | N | N | | **Pathology indicates bacterial Infection with a Gram-negative rod-shaped bacteria**; multiple granulomas in head & caudal kidney, mild branchitis, iritis, hepatitis. | N | |
| | | | | 11 | Spleen | N | | | N | | | N | | | | | |
| | | | | 12 | Brain | N | | | N | | | N | | | | | |
| | 5 | E | Stomach full | 13 | Kidney | + | P | | N | N | | N | N | | Mild Pathology: mild branchitis, iritis, pericarditis, mild branchitis,mild hepatitis. | N | |
| | | | | 14 | Spleen | N | | | N | | | N | | | | | |
| | | | | 15 | Brain | + | | | N | | | N | | | | | |
| | 6 | E | Stomach full, thin | 16 | Kidney | + | P | | N | N | | N | N | | **Moderate pathology**: mild branchitis, mild iritis, dermatitis, hepatitis, nephritis, inflammation of swim bladder. Moderate pericarditis. | N | |
| | | | | 17 | Spleen | + | | | N | | | N | | | | | |
| | | | | 18 | Brain | + | | | N | | | N | | | | | |
| R1 Low Dose (10$^3$ cfu/fish) | 1 | E | Stomach full | 1 | Kidney | N | P | 6/6+ve | N | N | 1/6 +ve | N | P | 5/6 +ve | **Marked pathology; Bacterial septicaemia: bacterial granulomas in spleen, caudal kidney, swim bladder. Mild branchitiis. Gram Glynn positive bacteria in kidney, granulomatous nephritis.** | P | 5/6 +ve |
| | | | | 2 | Spleen | N | | | N | | | N | | | | | |
| | | | | 3 | Brain | + | | | N | | | N | | | | | |
| | 2 | E | Stomach full, thin | 4 | Kidney | + | P | | N | N | | N | N | | Mild pathology; branchitiis. | N | |
| | | | | 5 | Spleen | + | | | N | | | N | | | | | |
| | | | | 6 | Brain | N | | | N | | | N | | | | | |
| | 3 | E | Stomach full, red caudal fin | 7 | Kidney | + | P | | + | P | | + | P | | **Moderate Pathology: GG positive bacteria brain, & pancreatic blood vessels**. Mild iritis, choroidits, branchitis, splenitis. | P | |
| | | | | 8 | Spleen | N | | | N | | | N | | | | | |
| | | | | 9 | Brain | + | | | N | | | N | | | | | |
| | 4 | E | Stomach full | 10 | Kidney | + | P | | N | N | | N | P | | **Mild-moderate pathology;**mild branchitiis, skeletal muscle fascitiis, pericarditis, hepatitis, splenitis (lots melanin scattered throughout spleen).**GG positive bacteria in meninges.** | P | |
| | | | | 11 | Spleen | N | | | N | | | N | | | | | |
| | | | | 12 | Brain | + | | | N | | | + | | | | | |
| | 5 | E | Stomach full, thin | 13 | Kidney | + | P | | N | N | | N | P | | **Mild pathology**; mild branchitiis, hepatitis, pericarditis.**GG positive bacteria seen in brain & sloughed macrophages between adjacent gill lamellae.** | P | |
| | | | | 14 | Spleen | + | | | N | | | N | | | | | |
| | | | | 15 | Brain | + | | | N | | | + | | | | | |
| | 6 | E | Stomach full, hyperaemic liver, | 16 | Kidney | + | P | | N | N | | N | P | | **Mild pathology**: branchitis, hepatitis, pericarditis, spletnitis. Lots large MMC in HK. **GG positive bacteria seen in meninges of brain.** | P | |
| | | | | 17 | Spleen | + | | | N | | | N | | | | | |
| | | | | 18 | Brain | + | | | N | | | + | | | | | |
| R1 Control | 1 | E | stomach half full | 1 | Kidney | N | N | 2/6+ve | N | N | 0/6 +ve | N | N | 0/6 +ve | Mild pathology; mild branchitiis. | N | 0/6 +ve |
| | | | | 2 | Spleen | N | | | N | | | N | | | | | |
| | | | | 3 | brain | N | | | N | | | N | | | | | |
| | 2 | E | stomach half full | 4 | Kidney | + | P | | N | N | | N | N | | **Moderate Pathology**: branchitiis. **Bacterial ifnection of kidney with Gram negative bacteria in granulomas,** granulomatous inflammation, hepatitis, inflamamtion of swim bladder connective tissue. | N | |
| | | | | 5 | Spleen | N | | | N | | | N | | | | | |
| | | | | 6 | brain | + | | | N | | | N | | | | | |
| | 3 | E | stomach full | 7 | Kidney | N | N | | N | N | | N | N | | **Moderate Pathology**: mild branchitiis, pericarditis of heart ventricle & atrium, large MMC in kidney. | N | |
| | | | | 8 | Spleen | N | | | N | | | N | | | | | |
| | | | | 9 | brain | N | | | N | | | N | | | | | |
| | 4 | E | stomach third full | 10 | Kidney | N | N | | N | N | | N | N | | Very mild pathology: mild branchitiis, a few melano-macrophages in head kidney (PHOTO), brain suspect. | N | |
| | | | | 11 | Spleen | N | | | N | | | N | | | | | |
| | | | | 12 | brain | N | | | N | | | N | | | | | |
| | 5 | E | stomach full | 13 | Kidney | N | N | | N | N | | N | N | | Mild Pathology: mild branchitiis, mild pericarditis & myocaridits of BA & ventricle, lots MMC in HK, mild hepatitis, lots melanin scattered throughout spleen. | N | |
| | | | | 14 | Spleen | N | | | N | | | N | | | | | |
| | | | | 15 | brain | N | | | N | | | N | | | | | |
| | 6 | E | stomach half full, red caudal fin | 16 | Kidney | N | P | | N | N | | N | N | | Mild to moderate Pathology: mild branchitiis,1 large bacterial granuloma in HK (ZN negative for mycobacteria), mild hepatitis, lots melanin scattered throughout spleen. Brain suspect (amorphous blue material in cells in meninges). | N | |
| | | | | 17 | Spleen | N | | | N | | | N | | | | | |
| | | | | 18 | brain | + | | | N | | | N | | | | | |
| R2 High Dose (10$^6$ cfu/fish) | 1 | EM | Disoriented,spiral swimming, thin,empty stomach, gall | 1 | Kidney | N | P | 5/6+ve | N | N | 2/6 +ve | N | P | **3/6 +ve** | **Marked pathology;** severe pericarditis, meningitis, hepatic necrosis, chroiditis, nephritis. **GG positive bacteria in brain, peritoneal cavity, heart** | P | **3/6 +ve** |
| | | | | 2 | Spleen | N | | | N | | | N | | | | | |
| | | | | 3 | Brain | + | | | N | | | + | | | | | |
| | 2 | E | Stomach full | 4 | Kidney | N | P | | N | N | | N | N | | **Moderate pathology**: branchitis, iritis, choroiditis,meningitis, pericarditis, nephritis, hepatitis, but no bacteria seen. | N | |
| | | | | 5 | Spleen | N | | | N | | | N | | | | | |
| | | | | 6 | Brain | + | | | N | | | N | | | | | |
| | 3 | E | Stomach full | 7 | Kidney | N | N | | N | N | | N | N | | **Mild to moderate pathology;** mild branchitis, iritis, choroiditis, pericarditis, endocarditis, hepatitis, nephritis, splenitis. | N | |
| | | | | 8 | Spleen | N | | | N | | | N | | | | | |
| | | | | 9 | Brain | N | | | N | | | N | | | | | |
| | 4 | E | Stomach full, red pectoral fin | 10 | Kidney | N | P | | N | P | | N | P | | **Moderate Pathology**; branchitis, meningitis, pericarditis, splenitis, nephrosis. **GG positive bacteria detected in brain.** | P | |
| | | | | 11 | Spleen | N | | | + | | | N | | | | | |
| | | | | 12 | Brain | + | | | N | | | + | | | | | |
| | 5 | E | Stomach full | 13 | Kidney | N | P | | N | N | | N | P | | **Moderate Pathology**; branchitis, choroiditis, meningitis, pericarditis, endocarditis, hepatitis, splenitis, nephrosis. **GG pos. bacteria detected in brain.** | P | |
| | | | | 14 | Spleen | N | | | N | | | N | | | | | |
| | | | | 15 | Brain | + | | | N | | | + | | | | | |
| | 6 | E | Stomach full | 16 | Kidney | + | P | | + | P | | N | N | | Mild pathology: mild branchitis, splenitis, myocarditis, pericarditis. | N | |
| | | | | 17 | Spleen | N | | | N | | | N | | | | | |
| | | | | 18 | Brain | N | | | N | | | N | | | | | |
| R2 Low Dose (10$^3$ cfu/fish) | 1 | E | Stomach full | 1 | Kidney | N | P | 6/6+ve | N | N | 0/6 +ve | N | N | **0/6 +ve** | Mild pathology; Mild branchitis, mild heaptitis, endocarditis, lots melanin in spleen, head kidney. | N | **0/6 +ve** |
| | | | | 2 | Spleen | N | | | N | | | N | | | | | |
| | | | | 3 | Brain | + | | | N | | | N | | | | | |
| | 2 | E | Stomach full | 4 | Kidney | N | P | | N | N | | N | N | | Mild pathology;mild hepatitis, lots melanin in spleen, head kidney. | N | |
| | | | | 5 | Spleen | N | | | N | | | N | | | | | |
| | | | | 6 | Brain | + | | | N | | | N | | | | | |
| | 3 | E | Stomach full | 7 | Kidney | N | P | | N | N | | N | N | | Mild pathology;mild choroiditis, branchitis, nephritis, hepatitis, lots melanin in spleen, head kidney. | N | |
| | | | | 8 | Spleen | N | | | N | | | N | | | | | |
| | | | | 9 | Brain | + | | | N | | | N | | | | | |
| | 4 | E | Stomach full | 10 | Kidney | N | P | | N | N | | N | N | | Mild pathology; mild choroiditis, iritis, branchitis, nephritis, hepatitis, lots melanin in spleen, head kidney. | N | |
| | | | | 11 | Spleen | N | | | N | | | N | | | | | |
| | | | | 12 | Brain | + | | | N | | | N | | | | | |
| | 5 | E | Stomach full | 13 | Kidney | N | P | | N | N | | N | N | | Very Mild Pathology: mild iritis (1 eye only). | N | |
| | | | | 14 | Spleen | N | | | N | | | N | | | | | |
| | | | | 15 | Brain | + | | | N | | | N | | | | | |
| | 6 | E | Stomach full | 16 | Kidney | N | P | | N | N | | N | N | | **Moderate pathology**: branchtiis, hepatitis, ganulomatous inflammation connective tissue of swim bladder, nephritis**:Gram-negative rod bacteria in central core of granulomas.** | N | |
| | | | | 17 | Spleen | N | | | N | | | N | | | | | |
| | | | | 18 | Brain | + | | | N | | | N | | | | | |
| R2 Control | 1 | E | stomach half full | 1 | Kidney | N | N | 1/6+ve | N | N | 0/6 +ve | N | N | **1/6 +ve** | Very mild iritis (1 eye only). | N | **1/6 +ve** |
| | | | | 2 | Spleen | N | | | N | | | N | | | | | |
| | | | | 3 | brain | N | | | N | | | N | | | | | |
| | 2 | E | stomach full | 4 | Kidney | N | N | | N | N | | N | N | | **Moderate pathology**:branchtiis, hepatitis, ganulomatous inflammation connective tissue of swim bladder, **nephritis; Gram-negative rod bacteria in central core of granulomas.** | N | |
| | | | | 5 | Spleen | N | | | N | | | N | | | | | |
| | | | | 6 | brain | N | | | N | | | N | | | | | |
| | 3 | E | stomach half full | 7 | Kidney | N | N | | N | N | | N | N | | Very mild iritis, mild pericarditis at apex of ventricle. Large melano-macrophage centres inhead kidney. | N | |
| | | | | 8 | Spleen | N | | | N | | | N | | | | | |
| | | | | 9 | brain | N | | | N | | | N | | | | | |
| | 4 | E | stomach full | 10 | Kidney | N | N | | N | N | | N | N | | Mild pathology: mild hepatitis | N | |
| | | | | 11 | Spleen | N | | | N | | | N | | | | | |
| | | | | 12 | brain | N | | | N | | | N | | | | | |
| | 5 | E | stomach full | 13 | Kidney | + | P | | N | N | | N | P | | **Moderate pathology** mild pericarditis & myocarditis of bulbus arterious, ventricle & atrium; **GG positive bacteria seen near AV valve in heart.** Head & caudal kidney have a few melano-macrophage centres. | P | |
| | | | | 14 | Spleen | N | | | N | | | N | | | | | |
| | | | | 15 | brain | N | | | N | | | N | | | | | |
| | 6 | E | stomach full | 16 | Kidney | N | N | | N | N | | N | N | | Mild branchitis only. | N | |
| | | | | 17 | Spleen | N | | | N | | | N | | | | | |
| | | | | 18 | brain | N | | | N | | | N | | | | | |

R1=Replicate 1; R2=Replicate 2; E=survivor, euthanased, EM=euthanansed, moribund. P, +ve=positive, N=negative, N/A= no sample available for testing.

# APPENDIX 8. COHABITATION CHALLENGE TRIAL (REPLICATE 1).

Summary of all laboratory test results for individual fish *E. lanceolatus* tested by PCR, bacteriology and histopathology analysis.

| Replicate & Treatment Group | Treat-ment | Fish No. | Fish Clinical signs | Fate of Fish | Fish sample No. | Fish Organ sampled | TAAHL PCR (individual organ) Result | TAAHL PCR (pooled) result | Total No. PCR Pos fish/ total no. tested | UQ Bacto (individual organ) result | UQ Bacto (pooled organ) result | Total no.Bacto Pos Fish/Total no. tested | TAAHL Gram Glynn (inidividual organ) result | TAAHL Gram Glynn (pooled organ) result | Total GG +ve fish/Total no. tested | TAAHL: Histopathology observation summary | Histology whole fish result | Total Histo +ve fish/Total no. tested |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| R1 Ratio 1:5 | I | 1 | stomach empty,dead, thin, small spleen, red brain | Dead | 2 | Kidney | N | P | 2/2+ve | N | N | 0/2 +ve | N/A | N/A | 1/2 +ve | **Post mortem degeneration (histology not interpretable)** | N/A | 1/2 +ve |
| | | | | | 3 | Spleen | N | | | N | | | N/A | | | | | |
| | | | | | 4 | Brain | + | | | N | | | N/A | | | | | |
| | I | 2 | stomach empty,on tank bottom, thin, very small liver | EM | 5 | Kidney | N | P | | N | N | | N | P | | Mild Pathology: mild choroiditis & irits, branchitis, pericarditis, meningitis. **Gram Glynn positive bacteria seen in brain, eyes.** | P | |
| | | | | | 6 | Spleen | N | | | N | | | N | | | | | |
| | | | | | 7 | Brain | + | | | N | | | + | | | | | |
| | NI | 1 | full stomach | E | 8 | Kidney | + | P | 6/6+ve | N | N | 0/6 +ve | N | N | 3/6 +ve | Mild pathology: dermatitis, mild myositis, v. mild hepatitis. No spleen. | N | 3/6 +ve |
| | | | | | 9 | Spleen | N | | | N | | | N/A | | | | | |
| | | | | | 10 | Brain | + | | | N | | | N | | | | | |
| | NI | 2 | full stomach, pectoral & caudal fin red | E | 11 | Kidney | N | P | | N | N | | N | P | | Mild pathology: mild branchtitiis, choroiditis, iritis, meningitis, dermatitis, v. mild hepatitis. **Bacteria seen in brain.** | P | |
| | | | | | 12 | Spleen | N | | | N | | | N | | | | | |
| | | | | | 13 | Brain | + | | | N | | | + | | | | | |
| | NI | 3 | full stomach,caudal fin red, hyperaemic liver | E | 2 | Kidney | + | P | | N | N | | N/A | N | | Mild pathology: mild choroiditis, iritis, peritonitis, pericarditis & endocarditis, mild hepatitis, spleen has many MMC & melanin | N | |
| | | | | | 3 | Spleen | + | | | N | | | N | | | | | |
| | | | | | 4 | Brain | + | | | N | | | N | | | | | |
| | NI | 4 | stomach half full, gall bladder full, caudal fin red | E | 5 | Kidney | + | P | | N | N | | N | P | | Mild to Moderate pathology: mild choroiditis, iritis, peritonitis, pericarditis & endocarditis, mild hepatitis, spleen & HK with lots melanin throughout. **A few Gram Glynn positive bacteria seen in choroid of eye & meninges of brain** | P | |
| | | | | | 6 | Spleen | + | | | N | | | N | | | | | |
| | | | | | 7 | Brain | + | | | N | | | + | | | | | |
| | NI | 5 | stomach half full | E | 8 | Kidney | + | P | | N | N | | N | P | | Mild to Moderate pathology: mild choroiditis, iritis, peritonitis, pericarditis & endocarditis, mild hepatitis. **A few A few Gram Glynn positive bacteria seen in menignes of brain.** | P | |
| | | | | | 9 | Spleen | + | | | N | | | N | | | | | |
| | | | | | 10 | Brain | + | | | N | | | + | | | | | |
| | NI | 6 | stomach, caudal fin red, liver hyerpaemic,mouth deformity | E | 11 | Kidney | + | P | | N | N | | N | N | | Mild to Moderate pathology: mild choroiditis, iritis, peritonitis, pericarditis & endocarditis, mild hepatitis | N | |
| | | | | | 12 | Spleen | + | | | N | | | N | | | | | |
| | | | | | 13 | Brain | + | | | N | | | N | | | | | |
| R1 Ratio 1:2 | I | 1 | stomach third full | E | 2 | Kidney | + | P | 5/5+ve | N | P | 1/5 +ve | N | P | 4/5+ve | **Marked pathology: choroditis, ophthalmitis, meningitis, peritonitis, enteritis, hepatitis,pericarditis, nephritis. A few Gram Glynn positive bacteria seen in brain, eyes.** | P | 4/5+ve |
| | | | | | 3 | Spleen | + | | | + | | | + | | | | | |
| | | | | | 4 | Brain | + | | | + | | | + | | | | | |
| | I | 2 | stomach empty, thin, found at bottom, small liver | EM | 5 | Kidney | + | P | | N | N | | + | P | | **Marked pathology: choroditis, ophthalmitis, meningitis, peritonitis, enteritis, hepatitis,pericarditis, nephritis. A few Gram Glynn positive acteria seen in brain, eyes.** | P | |
| | | | | | 6 | Spleen | + | | | N | | | + | | | | | |
| | | | | | 7 | Brain | + | | | N | | | + | | | | | |
| | I | 3 | stomach empty,thin, small liver | E | 8 | Kidney | + | P | | N | N | | + | P | | **Marked pathology: choroditis, ophthalmitis, meningitis, peritonitis, enteritis, hepatitis,pericarditis, nephritis. Gram Glynn positive bacteria seen in brain, eyes.** | P | |
| | | | | | 9 | Spleen | + | | | N | | | + | | | | | |
| | | | | | 10 | Brain | + | | | N | | | + | | | | | |
| | I | 4 | stomach full | E | 11 | Kidney | + | P | | N | N | | N/A | P | | **Marked pathology: choroditis, ophthalmitis, meningitis, peritonitis, enteritis, hepatitis,pericarditis, nephritis. Gram Glynn positive bacteria seen in brain, eyes.** | P | |
| | | | | | 12 | Spleen | + | | | N | | | N/A | | | | | |
| | | | | | 13 | Brain | + | | | N | | | + | | | | | |
| | I | 5 | stomach full, small spleen | E | 1 | Kidney | N | P | | N | N | | N | N | | Mild Pathology: mild iritis, branchitis,enteritis, spleen has lots MMC. | N | |
| | | | | | 2 | Spleen | N | | | N | | | N | | | | | |
| | | | | | 3 | Brain | + | | | N | | | N | | | | | |
| | NI | 1 | stomach third full | E | 14 | Kidney | + | P | 5/6+ve | + | P | 1/6 +ve | + | P | 1/6+ve | **Marked pathology: ophthalmitis, branchitis, meningitis, fascitis, hepatitis, peritonitis, pericarditis, splenitis. Gram Glynn positive bacteria visible in all organs.** | P | 1/6+ve |
| | | | | | 15 | Spleen | + | | | + | | | + | | | | | |
| | | | | | 16 | Brain | + | | | N | | | + | | | | | |
| | NI | 2 | stomach half full, pectoral & caudal fins red, lip deformity, liver discolored | E | 1 | Kidney | N | P | | N | N | | N | N | | Mild pathology but heart has endocarditis, with suspect cocci bacteria seen inside macrophages, HK & spleen have lots large MMC. | N | |
| | | | | | 2 | Spleen | + | | | N | | | N | | | | | |
| | | | | | 3 | Brain | + | | | N | | | N | | | | | |
| | NI | 3 | stomach third full, small liver | E | 4 | Kidney | N | N | | N | N | | N | N | | Mild pathology: eye has mild iritis, moderate pericarditis, spleen has lots MMC. | N | |
| | | | | | 5 | Spleen | N | | | N | | | N | | | | | |
| | | | | | 6 | Brain | N | | | N | | | N | | | | | |
| | NI | 4 | stomach half full,caudal fin red | E | 7 | Kidney | + | P | | N | N | | N | N | | Mild pathology: Spleen, Hk & Ck has large & many MMC. Mild pericaridtis | N | |
| | | | | | 8 | Spleen | + | | | N | | | N | | | | | |
| | | | | | 9 | Brain | + | | | N | | | N | | | | | |
| | NI | 5 | stomach third full, caudal fin slightly red | E | 10 | Kidney | N | P | | N | N | | N | N | | Mild pathology: mild iritis, mild enteritis, mild pericarditis & myocarditis Spleen, Hk, Ck has large & many MMC. | N | |
| | | | | | 11 | Spleen | + | | | N | | | N | | | | | |
| | | | | | 12 | Brain | + | | | N | | | N | | | | | |
| | NI | 6 | stomach empty, gall bladder full, caudal fin red | E | 13 | Kidney | + | P | | N | N | | N | N | | Mild pathology: Spleen, Hk & Ck has large & many MMC. Mild pericaridtis | N | |
| | | | | | 14 | Spleen | N | | | N | | | N | | | | | |
| | | | | | 15 | Brain | N | | | N | | | N | | | | | |
| R1 Ratio 1:1 | I | 1 | stomach half full | E | 2 | Kidney | + | P | 9/9+ve | + | P | 6/9 +ve | + | P | 9/9 +ve | **Marked pathology: choroditis, ophthalmitis, meningitis, peritonitis, enteritis, hepatitis, pericarditis, nephritis, splenitis, branchtiis. Gram Glynn positive bacteria seen in all organs & tissues.** | P | 9/9 +ve |
| | | | | | 3 | Spleen | + | | | N | | | + | | | | | |
| | | | | | 4 | Brain | + | | | + | | | + | | | | | |
| | I | 2 | stomach empty | E | 5 | Kidney | + | P | | + | P | | + | P | | **Marked pathology: choroditis, ophthalmitis, meningitis, peritonitis, enteritis, hepatitis, pericarditis, nephritis, splenitis, branchtiis. Gram Glynn positive bacteria seen in all organs & tissues.** | P | |
| | | | | | 6 | Spleen | + | | | + | | | + | | | | | |
| | | | | | 7 | Brain | + | | | + | | | + | | | | | |
| | I | 3 | stomach full, no ext signs | E | 8 | Kidney | + | P | | N | P | | N | P | | **Marked pathology: choroditis, ophthalmitis, meningitis, peritonitis, enteritis, hepatitis, pericarditis, nephritis, splenitis, branchtiis. Gram Glynn positive bacteria seen in all organs & tissues.** | P | |
| | | | | | 9 | Spleen | + | | | N | | | N | | | | | |
| | | | | | 10 | Brain | + | | | + | | | + | | | | | |
| | I | 4 | stomach half full | E | 11 | Kidney | + | P | | N | N | | N | p | | **Marked pathology: choroditis, ophthalmitis, meningitis, peritonitis, enteritis, hepatitis, pericarditis, nephritis, splenitis, branchtiis. Gram Glynn positive bacteria seen in all organs & tissues.** | p | |
| | | | | | 12 | Spleen | + | | | N | | | N | | | | | |
| | | | | | 13 | Brain | + | | | N | | | + | | | | | |
| | I | 5 | stomach half full | E | 14 | Kidney | + | P | | N | P | | N | P | | **Marked pathology: choroditis, ophthalmitis, meningitis, peritonitis, enteritis, hepatitis, pericarditis, nephritis, splenitis, branchtiis. Gram Glynn positive bacteria seen in all organs & tissues.** | P | |
| | | | | | 15 | Spleen | + | | | N | | | + | | | | | |
| | | | | | 16 | Brain | N | | | + | | | + | | | | | |
| | I | 6 | stomach full, no ext signs | E | 17 | Kidney | N | P | | N | P | | N | P | | **Marked pathology: choroditis, ophthalmitis, meningitis, peritonitis, enteritis, hepatitis, pericarditis, nephritis, splenitis, branchtiis.Gram Glynn positive bacteria in brain, eye choroid, dermis, heart (pricardium). Faint bluish material in macrophage in MMC in HK. (engulfed bacteria ??)** | P | |
| | | | | | 18 | Spleen | + | | | N | | | N | | | | | |
| | | | | | 19 | Brain | + | | | + | | | + | | | | | |
| | I | 7 | stomach empty | E | 20 | Kidney | + | P | | N | P | | N | P | | **Marked pathology: choroditis, ophthalmitis, meningitis, peritonitis, enteritis, hepatitis, pericarditis, nephritis, splenitis, branchtiis.Bacteria in brain, eye choroid, dermis, heart. Faint bluish materia seen inside macrophage within MMC in HK (engulfed bacteria ??)** | P | |
| | | | | | 21 | Spleen | N | | | N | | | + | | | | | |
| | | | | | 22 | Brain | + | | | + | | | + | | | | | |
| | I | 8 | stomach empty,right eye red,thin,small spleen | EM | 23 | Kidney | + | P | | N | N | | N | P | | **Marked pathology: severe ophthalmitis, meningitis, peritonitis, mild enteritis. Gram Glynn positive bacteria seen in brain, eye.** | P | |
| | | | | | 24 | Spleen | + | | | N | | | N | | | | | |
| | | | | | 25 | Brain | + | | | N | | | + | | | | | |
| | I | 9 | stomach empty, thin, small spleen,red brain | Dead | 26 | Kidney | + | P | | N | N | | N/A | P | | **Some Post Mortem degeneration of heart, liver , kidney such that histological interpretation of these organs not possible. Gram Glynn positive bacteria seen in brain.** | P | |
| | | | | | 27 | Spleen | + | | | N | | | N/A | | | | | |
| | | | | | 28 | Brain | + | | | N | | | + | | | | | |
| | NI | 1 | stomach half full | E | 29 | Kidney | N | P | 6/10+ve | N | N | 1/10 +ve | N/A | N | 0/10 +ve | Very mild pathology: branchitis, choroiditis,pericarditis, spleen congested. | N | 0/10 +ve |
| | | | | | 30 | Spleen | N | | | N | | | N | | | | | |
| | | | | | 31 | Brain | + | | | N | | | N | | | | | |
| | NI | 2 | stomach half full, no external signs | E | 32 | Kidney | N | N | | N | P | | N | N | | Mild Pathology: mild branchitis, spleen has lot of melanin pigment scattered throughout. | N | |
| | | | | | 33 | Spleen | N | | | N | | | N | | | | | |
| | | | | | 34 | Brain | N | | | + | | | N | | | | | |
| | NI | 3 | stomach full | E | 35 | Kidney | N | P | | N | N | | N | N | | Mild pathology: mild branchitis, heart has endocarditis, mild hepatitis,spleen has lots melanin scattered throughout. | N | |
| | | | | | 36 | Spleen | N | | | N | | | N | | | | | |
| | | | | | 37 | Brain | + | | | N | | | N | | | | | |
| | NI | 4 | stomach half full, no external signs | E | 38 | Kidney | N | P | | N | N | | N | N | | Mild Pathology: branchitis, heart has endocarditis & very mild pericarditis of V & BA, spleen has lots melanin scattered throughout. | N | |
| | | | | | 39 | Spleen | N | | | N | | | N | | | | | |
| | | | | | 40 | Brain | + | | | N | | | N | | | | | |
| | NI | 5 | stomach third full | E | 41 | Kidney | N | N | | N | N | | N | N | | Mild Pathology: mild branchitis. | N | |
| | | | | | 42 | Spleen | N | | | N | | | N | | | | | |
| | | | | | 43 | Brain | N | | | N | | | N | | | | | |
| | NI | 6 | stomach half full | E | 44 | Kidney | N | P | | N | N | | N | N | | Mild Pathology: mild choroiditis, spleen with mild, mild hepatitis. subcapsular oedema | N | |
| | | | | | 45 | Spleen | N | | | N | | | N | | | | | |
| | | | | | 46 | Brain | + | | | N | | | N | | | | | |
| | NI | 7 | NCXS | E | 47 | Kidney | + | P | | N | N | | N | N | | Mild pathology: mild branchitis, v. mild choroiditis & iritis (1 eye only); meningela blood vessls congested; swim bladder has one focal area inflammation of rete mirabile; atrium has one focal area inflammation, spleen has lots melanin scattered throughout organ. | N | |
| | | | | | 48 | Spleen | N | | | N | | | N | | | | | |
| | | | | | 49 | Brain | + | | | N | | | N | | | | | |
| | NI | 8 | NCXS | E | 50 | Kidney | + | P | | N | N | | N | N | | Mild pathology: mild branchitis, v. mild choroiditis & iritis (1 eye only); meningeal blood vessls congested; mild pericarditis, mild hepatitis, swim bladder has one focal area inflammation of rete mirabile, spleen has melanin pigment scattered throughout. | N | |
| | | | | | 51 | Spleen | N | | | N | | | N | | | | | |
| | | | | | 52 | Brain | + | | | N | | | N | | | | | |
| | NI | 9 | NCXS | E | 53 | Kidney | N | N | | N | N | | N | N | | Mild pathology: mild branchtiis, bacteiral gill disease ? Gram negative rod bacteria seen adhered to tips of many gill filaments. 1 deformed eye. | N | |
| | | | | | 54 | Spleen | N | | | N | | | N | | | | | |
| | | | | | 55 | Brain | N | | | N | | | N | | | | | |
| | NI | 10 | NCXS | E | 56 | Kidney | N | N | | N | N | | N | N | | Mild pathology: branchitis, very mild iritis (1 eye only); mild hepatitis. | N | |
| | | | | | 57 | Spleen | N | | | N | | | N | | | | | |
| | | | | | 58 | Brain | N | | | N | | | N | | | | | |
| R1 Control | C | 1 | NCXS | E | 1 | Kidney | N | N | 2/6 +ve | N | N | 0/6 +ve | N | N | 0/6 +ve | Mild pathology; mild branchitiis. | N | 0/6 +ve |
| | | | | | 2 | Spleen | N | | | N | | | N | | | | | |
| | | | | | 3 | Brain | N | | | N | | | N | | | | | |
| | C | 2 | NCXS | E | 4 | Kidney | + | P | | N | N | | N | N | | Moderate Pathology:branchitiis, Bacterial ifnection of kidney withGram negative bacteria in granulomas, granulomatous inflammation, hepatitis, inflamamtion of swim bladder connective tissue. | N | |
| | | | | | 5 | Spleen | N | | | N | | | N | | | | | |
| | | | | | 6 | Brain | + | | | N | | | N | | | | | |
| | C | 3 | NCXS | E | 7 | Kidney | N | N | | N | N | | N | N | | Moderate Pathology: mild branchitiis, pericarditis of heart ventricle & atrium, large MMC in kidney. | N | |
| | | | | | 8 | Spleen | N | | | N | | | N | | | | | |
| | | | | | 9 | Brain | N | | | N | | | N | | | | | |
| | C | 4 | NCXS | E | 10 | Kidney | N | N | | N | N | | N | N | | Very Mild Pathology: mild branchitiis, a few melano-macrophages in head kidney (PHOTO), brain suspect. | N | |
| | | | | | 11 | Spleen | N | | | N | | | N | | | | | |
| | | | | | 12 | Brain | N | | | N | | | N | | | | | |
| | C | 5 | NCXS | E | 13 | Kidney | N | N | | N | N | | N | N | | Mild Pathology: mild branchitiis, mild pericarditis & myocaridits of BA & ventricle, lots MMC in HK, mild hepatitis, lots melanin scattered throughout spleen. | N | |
| | | | | | 14 | Spleen | N | | | N | | | N | | | | | |
| | | | | | 15 | Brain | N | | | N | | | N | | | | | |
| | C | 6 | NCXS | E | 16 | Kidney | N | P | | N | N | | N | N | | Mild to moderate Pathology: mild branchitiis,1 large bacterial granuloma in HK (ZN negative for mycobacteria), mild hepatitis, lots melanin scattered throughout spleen.Brain suspect. | N | |
| | | | | | 17 | Spleen | N | | | N | | | N | | | | | |
| | | | | | 18 | Brain | + | | | N | | | | | | | | |

I=Injected; NI=Non-injected; E=Euthanased; EM=Euthanansed, moribund; S=Survivor;P, +ve=Positive: N=Negative; NCXS=No clinical signs.

# APPENDIX 9. COHABITATION CHALLENGE TRIAL (REPLICATE 2).

Summary of all laboratory test results for individual fish *E. lanceolatus* tested by PCR, bacteriology and histopathology analysis.

| Treat-ment | Fish No. | Fish Clinical signs | Fate of Fish | Sample No. | Organ tested | TAAHL PCR organ result | TAAHL PCR (pooled) Result | TAAHL Total PCR Pos. fish/no. tested | UQ Bacto (individual organ result) | UQ Bacto Result (pooled) | Total no. bacto Pos/total no. fish tested | TAAHL Gram Glynn (organ result) | TAAHL Gram glynn (pooled) result | Total no. GG +ve fish/total no. tested | TAAHL: Histopathology observation summary | TAAHL Histoloy whole fish result | Total no. Histo +ve fish/total no. tested |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| I | 1 | stom empty, splenomegally, skin discouloured | Dead | 2 | Kidney | + | P | 2/2 +ve | + | P | 1/2 +ve | + | P | 2/2 +ve | Marked pathology (Pathology as for as for injected fish previously described). **GG +ve Bacteria seen in brain, eyes.** | P | 2/2 +ve |
| | | | | 3 | Spleen | N | | | + | | | N | | | | | |
| | | | | 4 | Brain | + | | | + | | | + | | | | | |
| I | 2 | stom empty, erratic spiral swimming, big spleen | EM | 5 | Kidney | + | P | | N | N | | + | P | | Marked pathology (Pathology as for as for injected fish previously described). **Gram Glynn positive bacteria seen in brain, eyes.** | P | |
| | | | | 6 | Spleen | N | | | N | | | + | | | | | |
| | | | | 7 | Brain | + | | | N | | | + | | | | | |
| NI | 2 | full stomach, liver hyperaemic | E | 1 | Kidney | N | P | 5/6+ve | N | N | 0/6 +ve | N | P | 4/6 +ve | Mild pathology eye (iritis), brain (mild meningitis), **Gram Glynn positive bacteria seen in brain, eyes.** | P | 4/6 +ve |
| | | | | 2 | Spleen | + | | | N | | | N | | | | | |
| | | | | 3 | Brain | + | | | N | | | + | | | | | |
| NI | 3 | full stomach | E | 4 | Kidney | + | P | | N | N | | N | N | | Mild branchitiis, mild iritis | N | |
| | | | | 5 | Spleen | + | | | N | | | N | | | | | |
| | | | | 6 | Brain | N | | | N | | | N | | | | | |
| NI | 4 | full stomach, fins red | E | 7 | Kidney | N | N | | N | N | | N | P | | Mild branchitiis, mild iritis. GG +ve Bacteria seen in brain. | P | |
| | | | | 8 | Spleen | N | | | N | | | N | | | | | |
| | | | | 9 | Brain | N | | | N | | | + | | | | | |
| NI | 5 | stomach full, fin redish | E | 10 | Kidney | N | P | | N | N | | N | N | | Mild iritis, Endocarditis, myocarditis, fibrosis (unusual lesion). | N | |
| | | | | 11 | Spleen | N | | | N | | | N | | | | | |
| | | | | 12 | Brain | + | | | N | | | N | | | | | |
| NI | 6 | stom full, caudal fin red, mouth deformity | E | 13 | Kidney | N | P | | N | N | | N | P | | Mild branchitiis, iritis, keratitis, myocarditis. **Gram Glynn positive bacteriaa seen in brain.** | P | |
| | | | | 14 | Spleen | + | | | N | | | N | | | | | |
| | | | | 15 | Brain | + | | | N | | | + | | | | | |
| NI | 1 | full stomach | E | 8 | Kidney | + | P | | N | N | | N | P | | Mild pathology of heart (mild pericarditis, endocardosis, myocarditis), mild iritis. **Gram Glynn positive bacteria seen in heart.** | P | |
| | | | | 9 | Spleen | + | | | N | | | N | | | | | |
| | | | | 10 | Brain | + | | | N | | | N | | | | | |
| I | 1 | full stomach | E | 2 | Kidney | + | P | 4/5+ve | + | P | 2/5 +ve | + | P | 4/5 +ve | Marked pathology:choroditis, ophthalmitis, meningitis, peritonitis, enteritis, hepatitis, pericarditis, nephritis, splenitis, branchtiis. **GG +ve bacteria in gills, eye, brain, peritoneal cavity, kidney, spleen, liver, heart, pancreas**. | P | 4/5 +ve |
| | | | | 3 | Spleen | + | | | + | | | + | | | | | |
| | | | | 4 | Brain | + | | | + | | | + | | | | | |
| I | 2 | stomach empty, at bottom, spiral swimming, skin decolored | EM | 5 | Kidney | + | P | | N | N | | N | P | | Marked pathology: choroditis, ophthalmitis, meningitis, peritonitis, enteritis, hepatitis, pericarditis, nephritis, splenitis, branchtiis. **GG +ve bacteria in eyes (choroid), brain (3rd ventricle, meninges), peritoneal cavity, heart pericardium, liver.** | P | |
| | | | | 6 | Spleen | + | | | N | | | N | | | | | |
| | | | | 7 | Brain | + | | | N | | | + | | | | | |
| I | 3 | stomach full | E | 8 | Kidney | + | P | | N | P | | N | N | | Marked pathology (as above). G**G +ve bacteria visible in episcleral conenctive tissue of eye, but not in melanised granulomas in choroid**.Brain has dark blue amorphous material in meninges. | P | |
| | | | | 9 | Spleen | N | | | N | | | N | | | | | |
| | | | | 10 | Brain | N | | | + | | | N | | | | | |
| I | 4 | stomach half full | E | 1 | Kidney | N | P | | N | N | | N | P | | Marked pathology: choroditis, ophthalmitis, meningitis, peritonitis, enteritis, hepatitis, pericarditis, nephritis, splenitis, branchtiis. **GG +ve bacteria in gills, eyes (choroid), brain (3rd ventricle), fascia of muscel, peritoneal cavity.** | P | |
| | | | | 2 | Spleen | N | | | N | | | N | | | | | |
| | | | | 3 | Brain | + | | | N | | | + | | | | | |
| I | 5 | full stomach, liver discoloured | E | 4 | Kidney | N | N | | N | N | | N | N | | Pathology indicates granulomatous inflammatory infection, indicative fish may have overcome infection; lots melanin in MMC in spleen, HK, brain meninges, blood vessels, eye choroid. | N | |
| | | | | 5 | Spleen | N | | | N | | | N | | | | | |
| | | | | 6 | Brain | N | | | N | | | N | | | | | |
| NI | 1 | full stomach | E | 11 | Kidney | N | N | 3/6+ve | N | N | 0/6 +ve | N | N | 0/6 +ve | Very mild pathology: mild branchitis, choroiditis, iritis, hepatitis, peritonitis, myositis. Brain had meningitis but no bacteria visualised. | N | 1/6 +ve |
| | | | | 12 | Spleen | N | | | N | | | N | | | | | |
| | | | | 13 | Brain | N | | | N | | | N | | | | | |
| NI | 2 | stomach half full | E | 14 | Kidney | N | N | | N | N | | N | N | | Very mild pathology (v. mild branchitis, iritis, hepatitis). Abnormal, reduced size, inflamed swim bladder | N | |
| | | | | 15 | Spleen | N | | | N | | | N | | | | | |
| | | | | 16 | Brain | N | | | N | | | N | | | | | |
| NI | 3 | full stomach | E | 17 | Kidney | N | P | | N | N | | N | N | | Very mild pathology (v. mild branchitis, iritis, hepatitis). | N | |
| | | | | 18 | Spleen | + | | | N | | | N | | | | | |
| | | | | 19 | Brain | N | | | N | | | N | | | | | |
| NI | 4 | full stomach | E | 20 | Kidney | N | N | | N | N | | N | N | | Very mild pathology (v. mild branchitis, hepatitis, myositis ). | N | |
| | | | | 21 | Spleen | N | | | N | | | N | | | | | |
| | | | | 22 | Brain | N | | | N | | | N | | | | | |
| NI | 5 | full stomach, caudal fin red | E | 1 | Kidney | N | P | | N | N | | N | N | | Mild pathology of heart (pericarditis, endocarditis, myocarditis), inflammation swim bladder. | N | |
| | | | | 2 | Spleen | + | | | N | | | N | | | | | |
| | | | | 3 | Brain | + | | | N | | | N | | | | | |
| NI | 6 | stom full, caudal fin red, mouth deformity | E | 4 | Kidney | N | P | | N | N | | N | N | | Mild iritis. **Gram Glynn positive bacteria in eye choroid.** | P | |
| | | | | 5 | Spleen | + | | | N | | | N | | | | | |
| | | | | 6 | Brain | N | | | N | | | N | | | | | |
| I | 1 | stomach third full | E | 2 | Kidney | N | P | 10/10 +ve | N | N | 5/10 +ve | N | P | 10/10 +ve | Marked pathology. **Gram Glynn positive bacteria in choroid, peritoneal cavity, Intestinal lamina propria, pericardium.** | P | 10/10 +ve |
| | | | | 3 | Spleen | N | | | N | | | N | | | | | |
| | | | | 4 | Brain | + | | | N | | | + | | | | | |
| I | 2 | stomach third full | E | 5 | Kidney | + | P | | N | N | | + | P | | Marked pathology. **Gram Glynn positive bacteria in gills, eye conn tissue, meninges, peritoneal cavity, muscle, Intestinal lamina propria, pericardium of heart, MMC of spleen.** | P | |
| | | | | 6 | Spleen | + | | | N | | | + | | | | | |
| | | | | 7 | Brain | + | | | N | | | + | | | | | |
| I | 3 | stomach empty | E | 8 | Kidney | N | P | | N | N | | N | N | | Marked pathology. **Gram Glynn positive bacteria in gills, marked pericarditis of heart.** | P | |
| | | | | 9 | Spleen | + | | | N | | | N | | | | | |
| | | | | 10 | Brain | N | | | N | | | N | | | | | |
| I | 4 | stomach third full, L eye exophthalmos | EM | 11 | Kidney | + | P | | + | P | | + | P | | Marked pathology. **Gram Glynn positive bacteria in all organs & tissues.** | P | |
| | | | | 12 | Spleen | + | | | N | | | + | | | | | |
| | | | | 13 | Brain | + | | | + | | | + | | | | | |
| I | 5 | stomach third full | E | 14 | Kidney | + | P | | N | N | | N | P | | Marked pathology. **Gram Glynn positive bacteria in choroid of eye, brain meninges, dermis, heart.** | P | |
| | | | | 15 | Spleen | + | | | N | | | N | | | | | |
| | | | | 16 | Brain | + | | | N | | | + | | | | | |
| I | 6 | stomach third full | E | 17 | Kidney | + | P | | + | P | | N | P | | Marked pathology. **Gram Glynn positive bacteria in gills, eyes, brain, peritoneal cavity, heart pericardium, liver.** | P | |
| | | | | 18 | Spleen | + | | | + | | | N | | | | | |
| | | | | 19 | Brain | + | | | + | | | + | | | | | |
| I | 7 | stomach third full | E | 20 | Kidney | + | P | | N | N | | N | P | | Marked pathology. **Gram Glynn positive bacteria in choroid of eyes (marked choroiditis), brain meninges, peritoneal cavity, heart (pericardium).** | P | |
| | | | | 21 | Spleen | + | | | N | | | N | | | | | |
| | | | | 22 | Brain | + | | | N | | | + | | | | | |
| I | 8 | stomach third full, spiral swimming | EM | 23 | Kidney | + | P | | + | P | | N | P | | Marked pathology. **Gram Glynn positive bacteria in eyes, brain (meninges), heart (pericardium).** | P | |
| | | | | 24 | Spleen | N | | | N | | | N | | | | | |
| | | | | 25 | Brain | + | | | + | | | + | | | | | |
| I | 9 | stomach empty, thin, small liver & spleen. | Dead | 26 | Kidney | + | P | | N | P | | + | P | | Marked pathology. **Gram Glynn positive bacteria in eye (choroid), brain (meninges), head kidney, heart (pericardium), spleen.** | P | |
| | | | | 27 | Spleen | N | | | + | | | + | | | | | |
| | | | | 28 | Brain | + | | | + | | | + | | | | | |
| I | 10 | stomach third full | E | 29 | Kidney | + | P | | N | P | | N | P | | Marked pathology. **Gram Glynn positive bacteria in eye (choroid), brain (meninges), heart (pericardium), spleen.** | P | |
| | | | | 30 | Spleen | + | | | N | | | + | | | | | |
| | | | | 31 | Brain | + | | | + | | | + | | | | | |
| NI | 1 | full stomach | E | 32 | Kidney | N | N | 3/10+ | N | N | 1/10 +ve | N | N | 0/10 +ve | Very Mild pathology; mild branchitis, mild hepatitis. | N | 0/10 +ve |
| | | | | 33 | Spleen | N | | | N | | | N | | | | | |
| | | | | 34 | Brain | N | | | N | | | N | | | | | |
| NI | 2 | full stomach | E | 35 | Kidney | N | N | | N | P | | N | N | | Very Mild pathology; mild branchitis, mild hepatitis. | N | |
| | | | | 36 | Spleen | N | | | N | | | N | | | | | |
| | | | | 37 | Brain | N | | | + | | | N | | | | | |
| NI | 3 | full stomach | E | 38 | Kidney | N | N | | N | N | | N | N | | Very Mild pathology; mild branchitis, mild choroiditis, iritis, mild hepatitis, spleen ha lots melnin in lumen of splenic artery. | N | |
| | | | | 39 | Spleen | N | | | N | | | N | | | | | |
| | | | | 40 | Brain | N | | | N | | | N | | | | | |
| NI | 4 | full third full | E | 41 | Kidney | N | N | | N | N | | N | N | | Very Mild pathology; mild branchitis, mild hepatitis. | N | |
| | | | | 42 | Spleen | N | | | N | | | N | | | | | |
| | | | | 43 | Brain | N | | | N | | | N | | | | | |
| NI | 5 | stomach empty, right eye opacity, red caudal fins | EM | 44 | Kidney | N | N | | N | N | | N | N | | Very Mild pathology; mild branchitis, mild hepatitis, brain has dark blue staining material in meninges. | N | |
| | | | | 45 | Spleen | N | | | N | | | N | | | | | |
| | | | | 46 | Brain | N | | | N | | | N | | | | | |
| NI | 6 | stomach empty | E | 47 | Kidney | N | N | | N | N | | N | N | | Very Mild pathology; mild branchitis, mild hepatitis; swim Bladder abnormal, reduced in size; microsporidean-like parasite in spleen & head kidney haematopoietic tissue. | N | |
| | | | | 48 | Spleen | N | | | N | | | N | | | | | |
| | | | | 49 | Brain | N | | | N | | | N | | | | | |
| NI | 7 | N/A | E | 50 | Kidney | N | N | | N | N | | N | N | | Very Mild pathology; mild branchitis, mild hepatitis. | N | |
| | | | | 51 | Spleen | N | | | N | | | N | | | | | |
| | | | | 52 | Brain | N | | | N | | | N | | | | | |
| NI | 8 | N/A | E | 53 | Kidney | N | P | | N | N | | N | N | | Mild pathology; eye has retinal dysplasia, focal granulomatous myocarditis & pericarditis of heart atrium; mild dermatitis of fins; mild branchitis, mild hepatitis, focal skeletal muscle myositis. | N | |
| | | | | 54 | Spleen | + | | | N | | | N | | | | | |
| | | | | 55 | Brain | N | | | N | | | N | | | | | |
| NI | 9 | N/A | E | 56 | Kidney | N | P | | N | N | | N | N | | Very Mild pathology; mild branchitis, mild hepatitis, gills have nodular outgrowth of gill filament epidermal tissue. | N | |
| | | | | 57 | Spleen | N | | | N | | | N | | | | | |
| | | | | 58 | Brain | + | | | N | | | N | | | | | |
| NI | 10 | N/A | E | 59 | Kidney | + | P | | N | N | | N | N | | Very Mild pathology; mild branchitis, mild hepatitis, mild dermatitis. | N | |
| | | | | 60 | Spleen | N | | | N | | | N | | | | | |
| | | | | 61 | Brain | N | | | N | | | N | | | | | |
| C | 1 | None | E | 1 | Kidney | N | N | 1/6+ve | N | N | 0/6 +ve | N | N | 1/6 +ve | Very Mild Pathology: mild iritis (1 eye only). | N | 1/6 +ve |
| | | | | 2 | Spleen | N | | | N | | | N | | | | | |
| | | | | 3 | Brain | N | | | N | | | N | | | | | |
| C | 2 | None | E | 4 | Kidney | N | N | | N | N | | N | N | | Moderate pathology:branchitiis, hepatitis, ganulomatous inflammation connective tissue of swim bladder, nephritis (bacterial granulomas with Gram-negative rod bacteria | N | |
| | | | | 5 | Spleen | N | | | N | | | N | | | | | |
| | | | | 6 | Brain | N | | | N | | | N | | | | | |
| C | 3 | None | E | 7 | Kidney | N | N | | N | N | | N | N | | Very Mild Pathology: mild iritis, mild pericarditis at apex of ventricle. Large MMC in HK. | N | |
| | | | | 8 | Spleen | N | | | N | | | N | | | | | |
| | | | | 9 | Brain | N | | | N | | | N | | | | | |
| C | 4 | None | E | 10 | Kidney | N | N | | N | N | | N | N | | Mild pathology: mild hepatitis | N | |
| | | | | 11 | Spleen | N | | | N | | | N | | | | | |
| | | | | 12 | Brain | N | | | N | | | N | | | | | |
| C | 5 | None | E | 13 | Kidney | + | P | | N | N | | N | P | | Moderate pathology; mild pericarditis & myocarditis (bulbus arterious, ventricle & atrium). **Gram Glynn positive bacteria seen near AV valve in heart**. Head & caudal kidney:a few melano-macrophage centres. | P | |
| | | | | 14 | Spleen | N | | | N | | | N | | | | | |
| | | | | 15 | Brain | N | | | N | | | N | | | | | |
| C | 6 | None | E | 16 | Kidney | N | N | | N | N | | N | N | | Mild pathology: mild hepatitis | N | |
| | | | | 17 | Spleen | N | | | N | | | N | | | | | |
| | | | | 18 | Brain | N | | | N | | | N | | | | | |

I=Injected; NI=Non-Injected; C=Control; E=euthanased; EM=euthanansed, moribund; S=survivor; Pos, +ve=positive; N=negative, N/A= no sample available for testing.

# APPENDIX 10. QUEENSLAND GROUPER RISK ASSESSMENT

February 2011

## Executive summary

A workshop was conducted with a small group of scientists, veterinary pathologists and marine park managers to perform a risk assessment to:

1. Summarise and then rank (in order of impact) the current and potential hazards posed by the bacterium *Streptococcus agalactiae* to Queensland grouper (*E. lanceolatus*) and other fish species along the Queensland coastline; and
2. To identify and develop appropriate and sensible management responses to each hazard to reduce the risk.

The risk assessment indicated that high risks exist for the bacterium *Streptococcus agalactiae* to continue to cause lethal disease in Queensland grouper and for the bacterium to spread along the Queensland coast to infect other Queensland grouper in near shore habitats. The risks of the bacterium causing lethal disease in other fin-fish species in the near shore habitats and this leading to flow-on impacts for commercial and recreational fisheries and the ecosystem was also considered to be 'high'. In addition, the other area where the risk was considered to be 'high' was for the bacterium to enter (via translocation of an infected fish) and cause significant mortalities and fish-kills in marine aquaria and commercial marine aquaculture facilities.

The primary factor limiting informed decision making about how to respond to this situation is a lack of basic information about the biology, behaviour, ecology, stock structure and population status of Queensland grouper in near shore habitats combined with a lack of understanding of the aetiology and pathogenesis of *S. agalactiae* infections in these fish. This lack of information confounded the effectiveness of applying management controls to reduce risk.

The primary practical management controls that could be implemented to reduce these 'high risk' hazards and improve our understanding of the disease were largely about gaining a better understanding of the issue by supporting the existing carcass response program; supporting an existing *Fisheries Research & Development Corporation* (FRDC) project on the aetiology of the bacterium and risks to Queensland grouper and advising the marine aquaculture and marine aquarium industries about the risks of translocating high-risk fish (or rays) that could be infected with *S. agalactiae* into their facilities and the potential for subsequent fish kills. In addition, incorporating elements of this research into a more coordinated study on inshore biodiversity would be advantageous.

Based on this outcome no additional work, apart from those existing programs outlined above, is proposed at this stage.

**Background**

Since October 2007 there have been ~78 (confirmed and unconfirmed) Queensland Grouper (*E. lanceolatus*) carcasses reported from near shore waters from Northern and central Queensland. Three animals from reefal or offshore waters were reported (one from Lizard Island; one from Pixie Reef and one from Hardy Reef). Nearly all the carcasses were large giant grouper more than 1m long and more than 10 years old (based on sectioning otoliths from a limited number of specimens).

There were a high number of mortalities in early 2008 which prompted Biosecurity Queensland to form the *'Queensland Grouper Mortality Investigation Taskforce* (QGMITF)' with representatives from the Queensland Department of Primary Industries and Fisheries, Queensland Boating and Fisheries Patrol, Queensland Environmental Protection Agency, Cairns Regional Council, Cairns Port Authority, Cairns Marine Aquarium and the Great Barrier Reef Marine Park Authority. This taskforce was formed to advise the Queensland government on the disease outbreak and a sub-group of the QGMITF, the Technical Working Group (TWG), was also established to provide technical advice and to develop a strategy to examine suitable actions to better understand the issue and mitigate the risks.

Of those Queensland grouper carcasses fresh enough to sample, the bacterium *Streptococcus agalactiae* was identified and implicated as causing the mortality of the majority of the animals through bacterial septicaemia (blood poisoning). These were the first reported cases of streptococcosis due to *S. agalactiae* in wild fish in Queensland, and at the time, of any wild native fish in Australia. This appeared to be a newly emerging bacterial disease of Queensland grouper and other marine fish species. Twelve healthy wild fish from Trinity Inlet were sampled, and the bacterium was isolated from one live mullet (*Mugil* sp.). The bacterium was also isolated from a moribund wild fork-tailed catfish (*Arius thalassinus*) and two sick javelin fish (*Pomadasys kaakan*) in 2009. In 2009, and again in 2010, the bacterium caused disease epizootics in captive stingrays (Order Rajiformes), and was isolated from live and dead stingrays from a large commercial marine aquarium facility, *Sea World* in south east Queensland. In the 2009 disease epizootic, animals become moribund and some died following the introduction of wild animals from a marine aquarium supplier.

A draft strategy was produced by the technical working group to provide a prioritised framework for better understanding the epidemiology and pathogenesis of *S. agalactiae* (Attachment 1). This strategy was based around a sampling strategy including a mixture of laboratory and field-based research and monitoring. However, following a dramatic reduction in the number of Queensland grouper mortalities reported in mid-2009, the QGMITF was disbanded. The draft strategy was never formally accepted and implemented.

Between October 2007 and February 2010 the majority of Queensland grouper carcasses were reported from the area between Trinity Inlet and the Daintree River. Since April 2010, however, six carcasses were reported from the Gulf of Carpentaria, nine from the Townsville/Ingham area; one from Yeppoon, Central Queensland, one from Hardy Reef in the Whitsunday Island group, and one from the Brisbane River in southeast Queensland. Three carcasses were reported from the Cairns-Port Douglas area during 2010. There have also been confirmed and unconfirmed reports of Queensland grouper carcasses from waterways from the Gulf of Carpentaria, and a positive identification of *S. agalactiae* from a carcass sampled from a beach near Kowanyama in the Gulf of Carpentaria.

There has been no formal risk assessment conducted on the potential risks posed by the bacterium *Streptococcus agalactiae* that has caused infection and death in Queensland grouper and other fish species. There are concerns that the focal point of the bacterial infection has moved southwards (from around Cairns to around Townsville), and there is potential for the infection to spread to other commercially-important finfish species and into aquaculture facilities. This could have impacts on some of the social values of the Great Barrier Reef, on the livelihoods of commercial fishers, fish farmers and the dive-based tourism industry that rely on healthy fish stocks and on the individual species and the ecosystem. A risk assessment is therefore a logical extension of on-going work to identify the hazards and provide practical management controls to reduce or mitigate the risk.

**Issues and sensitivities**

These mortalities have been having an obvious and very public impact on large, iconic fish from within the Great Barrier Reef Marine Park (GBRMP). Given that these fish are high-order predators and are likely to be present in inshore habitats in low numbers, it is possible the removal of large, mature individuals may have impacts on the Queensland grouper population directly and with flow-on effects to the ecosystem. It is possible (but unknown) whether there is an element of cryptic mortality for smaller fish species, but these may not be recorded because they are being predated/scavenged by larger predators and hence not reported.

Given that *S. agalactiae* has been identified as causing streptococcosis in javelin fish (*Pomadasys kaakan*) from Trinity Inlet, there are concerns that *S. agalactiae* could spread to other fish species, especially important inshore species like barramundi (*Lates calcarifer*) and threadfin salmon (blue threadfin *Eleutheronema tetradactylum* and king threadfin *Polydactylus macrochir*). There are also concerns because streptococcal infections in marine mammals can occur after ingestion of prey items containing streptococcal organisms. For example, *S. agalactiae* was isolated from the internal organs of a dead bottlenose dolphin (*Tursiops truncatus*) that had ingested large numbers of mullet infected with *S. agalactiae* (the mullet *Liza klunzingeri*, were from the fish kill in Kuwait Bay) (Evans *et al.* 2008; 2009). There are concerns about the potential translocation of the bacterium between marine aquaria supply facilities and receiving facilities, as was evidenced in the above-mentioned situation with the stingrays.

*S. agalactiae* has caused large-scale wild fish kills in several countries including the USA, Kuwait and Israel following exposure of the fish to extreme environmental conditions (Plumb *et al.* 1974; Baya *et al.* 1990; Al-Marzouk *et al.* 2005; Evans *et al.* 2006). Based on this information and the description of *S. agalactiae* as a pathogen of farmed tilapia, there is the possibility for the bacterium to enter and cause similar disease in aquaculture facilities along the Queensland coast. This is especially concerning for those businesses that source wild-caught broodstock from within the GBRMP.

There are on-going resourcing implications for maintaining a response to examine and collect samples from carcasses of dead Queensland grouper.

**Review of *S. agalactiae* and infections by this bacterium**

*S. agalactiae* is a bacterial pathogen that causes significant disease in terrestrial animals including mastitis in cows and neonatal meningitis in newborn humans. It can cause a wide spectrum of diseases in immuno-compromised humans and is considered a potential emerging pathogen. In marine mammals and captive juvenile saltwater crocodiles it has caused necrotizing fasciitis and septicaemia. In fish erratic swimming, whirling, 'C' shaped curvature of the spine, external lesions of

haemorrhage, corneal opacity or exophthalmia (bulging eyes) and bacterial septicaemia have been reported.

Although streptococcosis caused by *S. agalactiae* has been described from farmed fish, emerging as a disease of tilapia, fish kills affecting estuarine and marine fish in natural systems caused by *S. agalactiae* have largely been observed in the USA (Chesapeake Bay, Alabama and Florida coast), Kuwait and Israel.

In 1972 *S. agalactiae* (non-haemolytic) was isolated from 90% of fish from large fish kills in estuaries in the Alabama and Florida coasts during the summer of 1972 (Plumb *et al*. 1972).

In the summer of 1988 mortalities of bluefish (*Pomatonus saltatrix*), striped bass (*Morone saxatilis*) and sea trout (*Cynoscion regalis*) occurred in Chesapeake Bay (Baya *et al*. 1990). Fish were lethargic, unresponsiveness to capture, had haemorrhage at the base of the fins, mouth and operculum, exophthalmia, corneal opacity and occasionally abdominal distension. Internally fish had ascites, pale, mottled or red livers, enlarged dark red spleens and cloudy meninges and cerebro-spinal fluid. Histological findings included inflammation and haemorrhage in the eye, chronic inflammation in the optic nerve sheaths and meninges of the brain, chronic epicarditis, kidney necrosis, and depleted lymphocytic and lymphoblastic cells in the spleen. *S. agalactiae* (non-haemolytic) was isolated and was the same strain as that isolated from the 1972 outbreak. High water temperatures and high salinities during summer were factors thought to predispose fish to infection (Baya *et al*. 1990).

In 2001 in Kuwait Bay a large fish kill involving 2100 t consisting of 99 per cent wild mullet (*Liza klunzingeri*) and 1 per cent silvery croaker, giant sea catfish and striped grunt occurred from August to mid-September and was caused by a (ß-haemolytic) *S. agalactiae.* Fish showed erratic swimming, haemorrhages around the mouth, abdomen, and fins and exophthalmia (inflammation of the eye). The outbreak coincided with a period of high sea-water temperatures (32.5-36.4°C) and high salinities (43 ppt) while dissolved oxygen levels ranged from 3.-7.0mg/l (Al-Marzouk *et al*. 2005).

Streptococcal infections in marine mammals can occur after ingestion of materials or prey items containing streptococcal organisms. For example, *S. agalactiae* was isolated from internal organs of a dead dolphin that had ingested large numbers of mullet infected with *S. agalactiae* (the mullet were from the fish kill in Kuwait Bay).

Sewage may be a source of infection. The *S. agalactiae* isolated from two sewage outfalls adjacent to beaches where fish kills occurred in Kuwait bay was identical to the fish isolate, and suggests sewage is a possible source of contamination to fish.

**Risk assessment approach**

A workshop was held at the Great Barrier Reef Marine Park Authority (GBRMPA) in Townsville on 20 July 2010. This meeting was attended by representatives from the GBRMPA (Mark Read, Adam Smith, Darren Cameron, John Tapim, Rachel Pears, Tyrone Ridgeway, and Randall Owens) and Veterinary Pathologists from Biosecurity Queensland (Dr Ian Anderson and Dr Rachel Bowater). Representatives from the Department of Environment and Resource Management (DERM) and the James Cook University's Fish and Fisheries Team were invited but were unable to attend.

The aim of the risk assessment workshop was two-fold.

1. Firstly to summarise and then rank (in order of impact) the current and potential hazards posed by *S. agalactiae* to the:

- Inshore population of Queensland grouper directly;
- To the ecosystem including other species of fish that may be infected or act as reservoirs for the bacteria; and
- The industries that rely on the marine ecosystem where the mortalities of the Queensland grouper have been recorded (such as marine aquaria, marine aquaculture and commercial fisheries).

2. Secondly, to identify and develop appropriate and sensible management responses to each hazard to reduce the risk.

Dr Rachel Bowater from Biosecurity Queensland provided a comprehensive background presentation on the history and aetiology of the infection of Queensland grouper by *S. agalactiae*, and also outlined the research she will be doing under a recent project funded by the Fisheries Research Development Corporation (FRDC). The objectives of Dr Bowater's FRDC project are to combine elements of laboratory- and field-based research to:

1. Perform a challenge infectivity trial in Queensland grouper to prove experimentally that *S. agalactiae* causes mortalities in Queensland grouper (or barramundi) to fulfil Koch's postulates;
2. Develop reliable, rapid and accurate diagnostic tools to enable detection of the bacterial disease streptococcosis caused by *S. agalactiae* in marine fish;
3. To perform phylogenetic comparison of Australian fish, human and animal strains of *S. agalactiae* with overseas fish and animal *S. agalactiae* strains, to determine their genetic relatedness and origin of the grouper strain (introduced or endemic);
4. To create a library of different *S. agalactiae* strains enabling utilisation by scientific researchers;
5. Determine the presence of *S. agalactiae* in a range of marine food species that are potentially a source of infection for Queensland grouper in the Cairns region;
6. Determine the prevalence of *S. agalactiae* in selected marine food sources for Queensland grouper in Cairns region, and
7. Determine the distribution and prevalence of *S. agalactiae* in fish along the North Queensland coast.

It should be noted that many of the objectives outlined above are similar to those in the draft strategy produced by the Queensland Grouper Technical Working Group (see Attachment 1).

**Identifying the hazards**

It has to be recognised that for this risk assessment there are many elements where this is limited or no data available upon which to inform decision making. This necessitated that the project team had to make some assumptions and decision-rules to assist with the process.

The project team considered that the major hazards posed by the emerging disease risk of infections of *S. agalactiae* in Queensland Grouper and other fish species could be categorised as:

- Direct impact of the bacterium on Queensland Grouper and other species;
- Impact on the ecosystem;
- Impact on marine industries and aquaculture; and
- Impact on human health.

Within each of these categories the project team considered the impacts of each hazard on the species and/or taxa, flow-on effects to the ecosystem; social and economic impacts and whether there were data deficiencies that required further information. Each hazard was ranked against standard likelihood and consequence tables (Attachment 2).

**Results**

The results of the of the risk assessment (Attachment 3) indicate that high risks exist for *S. agalactiae* to continue to cause lethal disease in Queensland grouper and for the bacterium to spread along the Queensland coast to infect other Queensland grouper in near shore habitats. The risks of the bacterium causing lethal disease in other fin-fish species in the near shore habitats and this leading to flow-on impacts for commercial and recreational fisheries and the ecosystem were also considered to be 'high'. The other area where the risk was considered to be 'high' was for the bacterium to enter and cause significant mortalities and fish-kills in marine aquarium and commercial marine aquaculture facilities.

One of the primary factors limiting informed decision making about how to respond to this situation is a lack of basic information about the biology, behaviour, ecology, stock structure and population status of Queensland grouper in near shore habitats combined with a lack of understanding of the aetiology and pathogenesis of *S. agalactiae* infections in these fish. This hampered the ability of the project team to make decisions about where to prioritise resources within the GBRMP and to understand the aetiology and pathogenesis of the disease. There is a lack of information to address fundamental questions such as:

- Whether the bacterium could spread between fish and along the coastline?;
- What is the mechanism for disease transmission?;
- Does a significant reservoir of the bacterium already exist in Queensland grouper and other species?, and
- Whether a lethal disease outbreak was triggered by some form of environmental change?

As indicated in Attachment 3 the primary practical management controls that could be implemented to reduce these 'high risk' hazards and improve our understanding of the disease were largely about gaining a better understanding of the issue by supporting the existing carcass response program; supporting Dr Rachel Bowater's existing FRDC project and advising the marine aquaculture and marine aquarium industries about the risks of translocating high-risk fish (or rays) that could be infected with *S. agalactiae* into their facilities and the potential for fish kills. In addition, incorporating elements of this research into a more coordinated study on inshore biodiversity would be advantageous.

Based on this outcome no additional work, apart from those existing programs outlined above, is proposed at this stage.

## Attachment 1.

**Further investigations in the epidemiology and pathogenesis of *S. agalactiae*.**
**Queensland Grouper Technical Working Group.**

**Draft: 15 June 2009**

**Preamble:**

The Technical Working Group still feels we need to better understand why adult-sized QUEENSLAND grouper were susceptible to infection by *S. agalactiae*? Why was there a large spike of grouper mortalities in early-mid 2008 that hadn't been recorded previously and have not been recorded since?

This draft strategy represents the first set of questions to be addressed. It has been divided into first and second tier questions to provide a prioritised approach to resource allocation and investment. The strategy will need to be amended or new strategies developed as new information/new findings comes to light.

<u>**TIER 1**</u>

1. **Proactive sampling to determine the prevalence of *S. agalactiae* in wild marine fish in the Cairns/Trinity Inlet area.**

**Aim:** To determine the prevalence of *S. agalactiae* as a pathogen in wild marine fish in the Cairns/Trinity Inlet area. To date we have *S. agalactiae* recorded from four species (Queensland Grouper, Grunter, mullet and fork-tailed catfish) sampled/retrieved from the Cairns/Trinity Inlet area, indicating that this area is a 'hotspot' for the bacterial infection. What we don't understand is whether the bacteria is prevalent in a small or large proportion of the population?

**Materials and methods:**

a) Site selection for targeted surveillance study: suggest tidal flats of Trinity Bay and the estuaries of Trinity Inlet
b) Target a statistically robust sample (suggest 40-60 individuals per species; <u>seek input from a biometrician re sample size and experimental design</u>) of three species (grunter, mullet and catfish) at both these sites and target organ(s) for testing.
c) May need to stratify the sampling regime to be able to pick up seasonal trends (if these exist?)
d) Use these data to determine the prevalence of *S. agalactiae* in the wild population of these fish species.

2. **Proactive sampling to determine possible sources of contamination for those fish species testing positive to *S. agalactiae* in the Cairns/Trinity Inlet area**

**Aim:** To further understand whether those fish species that are testing positive to *S. agalactiae* are contracting the infection via a food source

a. Is *S. agalactiae* present in the food items targeted by Queensland Grouper, grunter and catfish?

**Materials and methods:**

a) Site selection for targeted surveillance study: suggest focussing sampling effort in Trinity Bay and the estuaries of Trinity Inlet.
b) Target a limited number (suggest five to ten per species) of a broad range of possible food source species (suggest mud herring, mud crabs and prawns).

3. **Maintain passive sampling regime of sick or freshly dead fish.**

**Aim:** To collect additional samples (opportunistically) from sick or freshly dead fish reported between Cooktown and Bowen (even further afield?) and test for the presence of *S. agalactiae*. Also important to

continue to collect basic morphological data to better understand the changes in body condition of the infected fish.

**Materials and methods:** As per already developed protocols.

**Q:** How to we access carcasses from locations outside the Cairns region? We need to develop a sampling program with assistance from DERM and QBFP in areas outside the Cairns region so that they will pick this us as part of their commitment to the investigation.

**TIER 2**

**4. Proactive sampling to determine the distribution of *S. agalactiae* in North Queensland marine finfish.**

**Aim:** To further understand the role of *S. agalactiae* as a pathogen in wild marine fish in selected sites across Northern Queensland. To address this aim, two fundamental questions arise:

a. Is *S. agalactiae* a recent introduction into the area, and are fish developing clinical disease (following infection) because they are immunologically naive (i.e. have no previous exposure to the pathogen and therefore have no resistance)?
b. Is *S. agalactiae* an endemic (naturally occurring) infection in wild marine finfish? If so, then what predisposing host or environmental factors/stressors are affecting fish in the NQ region to allow the infection to develop into a systemic, clinical disease?

The important first step is to determine how widespread *S. agalactiae* infections are in fishes of North Queensland. This can be achieved by a targeted sampling study to detect the presence/absence of *S. agalactiae* in a variety of clinically normal fish in several distinct geographical locations. These studies will assist us in determining how widespread the pathogen is in native marine fishes of North Queensland.

Results from these studies will help determine the direction of future research. If *S. agalactiae* is widespread (i.e. endemic in fish populations along coastal regions of North Queensland), then research can then focus on other factors that may be predisposing fish to the bacterial infection. Alternatively, if the pathogen appears to be isolated to specific geographical regions, it is possible the pathogen may have been introduced, so future research will focus on identifying possible sources of infection, and how the pathogen is spread.

**Materials and methods:**

a) Test sensitivity to detect carrier status:
    i. Implement/develop real-time *S. agalactiae* PCR and compare to primary isolation on blood agar (BA) plates and selective broth enrichment and then isolation on BA plates.
b) Site selection for targeted surveillance study: suggest Port Douglas/Dicksons Inlet, Cairns/Trinity Inlet, Cardwell and Townsville.
c) Target a limited number (suggest five to ten per species) of a broad range of fish species (include species tested in point 1 above; suggest concentrate effort on benthic species including stingrays) and target organ(s) for testing.

**5. Experimental transmission trials to determine the infectious dose and route of infection.**

The risk of *S. agalactiae* to the aquaculture industries of North Queensland is currently unknown. The aquaculture farming industry in Northern Queensland has a current estimated worth of $49 million. Further studies are required to determine the pathogenicity of *S. agalactiae* to barramundi, *Lates calcarifer*, and reef fish species currently being cultured; Queensland grouper *E. lanceolatus*, gold spot cod *E. coioides*, coral trout, *Plectropomus leopardus*, barramundi cod *Cromileptes altivelis* and other species.

**Justification:** Monitoring and laboratory testing of clinically abnormal sick or dying Queensland grouper, grunter and catfish from Port Douglas and Cairns demonstrated *S. agalactiae* was often associated and was a lethal pathogen of fish. One healthy, clinically normal fish, a diamond scale mullet, also had *S. agalactiae* isolated from the heart. The original source of *S. agalactiae*, the method of transmission of *S. agalactiae* (and if fish-to-fish transmission occurs) is currently unknown. Consequently wild fish carrying *S. agalactiae* (*S. agalactiae* carriers) may be a source of infection for cultured marine finfish in Cairns. *S. agalactiae* carriers may

also be responsible for spreading the infection to Queensland grouper, grunter, catfish and other fish species in the Cairns inlet. Information on the pathogenicity and virulence of *S. agalactiae* on candidate cultured marine finfish is required to assist in the formation of disease risk assessments, and planning on-farm biosecurity and control measures required in the face of disease epizootics on fish farms. From our findings so far, and the information published overseas, it is clear *S. agalactiae* is pathogenic to both wild and cultured fish. However, currently we do not know the route of infection and whether *S. agalactiae* is a primary pathogen in the true sense i.e., whether or not low infectious doses invariably lead to clinical disease.

**Issues:**

a) Maintaining wild-type virulence of clinical *S. agalactiae* isolates.
b) No $LD_{50}$ experiments. Identify first signs of infection for point of euthanasia.
c) Infectious dose for parental injection, *per os* and in bath exposure
d) Experimental fish.

**6. A desktop analysis of contaminant sources (contemporary and historical)/water quality data/rainfall patterns and flood events/water temperature data for Trinity Inlet and Dicksons' Inlet to see if there are some indications as to why the outbreak occurred in mid-2008.**

**Aim:** To conduct a detailed desktop analysis of relevant environmental and biophysical/biochemical data for the Cairns region to see if there is some indicator(s) that will help us to better understand the pattern of mortalities. NB: These data would need to be compiled and analysed by someone with relevant experience in this field, such as an environmental scientist. We are not sure if this expertise sits within the Technical Working Group at present, so we may therefore need to look further afield to resource this.

**7. Work with recreational fishers through the AustFish program to get data on distribution, growth and movements of juvenile-adult sized Queensland grouper.**

Given that Queensland grouper represent the most significant group impacted by *S. agalactiae*, it would be advantageous for the Taskforce to have better information on the distribution/abundance, growth and movement patterns of juvenile to adult sized fish. Recreational fishers via the AustFish tagging program are capturing, measuring and tagging Queensland grouper as part of this program. There are some 140 Queensland grouper tagged and there have been some recaptures as well. The Taskforce would benefit by analysing these data and including the information into its database/GIS maps.

It is suggested that the Taskforce considers providing minor support to the AustFish group (suggest purchase of 1000 fish tags @ ~$700) to facilitate the on-going provision of data on captures/recaptures of Queensland grouper.

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## Attachment 2.

**Criteria for ranking likelihood and consequence to Queensland grouper, other species, marine-based industries and human health from lethal and non-lethal infection from the bacterium *S. agalactiae*.**

**Table 1. Consequence table.**

| DESCRIPTION | DEFINITION |
|---|---|
| CATASTROPHIC | Impact is clearly affecting the nature of the ecosystem over a wide area OR impact is catastrophic and possibly irreversible over a small area or to a sensitive population or community. Recovery periods of greater than 20 years likely OR condition of an affected part of the ecosystem irretrievably compromised. |
| MAJOR | Impact is significant at either a local or wider level or to a sensitive population or community. Recovery periods of 10 - 20 years are likely. Widespread impacts on industries/widespread social concern. |
| MODERATE | Impact is present at either a local or wider level. Recovery periods of 5 - 10 years anticipated. |
| MINOR | Impact is present but not to the extent that it would impair the overall condition of the ecosystem, sensitive population or community in the long term. |
| INSIGNIFICANT | No impact or, if impact is present, then not to an extent that would draw concern from a reasonable person. No impact on the overall condition of the ecosystem. |

**Table 2. Likelihood Table.**

| DESCRIPTION | FREQUENCY | PROBABILTY |
|---|---|---|
| ALMOST CERTAIN | Expected to occur more or less continuously throughout a year (e.g. more than 250 days per year) | 95-100% chance of occurring |
| LIKELY | Expected to occur once or many times in a year (e.g. 1 to 250 days per year) | 71-95% chance of occurring |
| POSSIBLE | Expected to occur once or more in the period of 1 to 10 years | 31-70% chance of occurring |
| UNLIKELY | Expected to occur once or more in the period of 10 to 100 years | 5-30% chance of occurring |
| RARE | Expected to occur once or more over a timeframe greater than 100 years | 0-5% chance of occurring |

**Attachment 3. Risk Assessment Table.**

| Activity or element | Hazard | Factors | Initial risk | Proposed treatment/management | Post management risk |
|---|---|---|---|---|---|
| Impact on Marine Industries and Aquaculture | Direct threat posed by lethal disease outbreaks of *S. agalactiae* in Queensland Grouper and other species impacting the GBR tourist industries, recreational interests, commercial fisheries, aquaculture, aquarium supply and display facilities, traditional fishing. | Impact on the social amenity of the Great Barrier Reef and direct impacts on the economics of the industries that rely on this ecosystem. | Major x Possible = High | • Support for Dr Bowater's FRDC project to improve knowledge and understanding<br>• Maintain reporting network and on-going response to Queensland Grouper carcasses<br>• Clarify responsibilities and who responds to grouper carcasses | Unsure because suggested management controls are about increasing understanding and awareness, not directly related to reducing risk. |
| Impact on Marine Industries and Aquaculture | Risk of *S. agalactiae* making its way into commercial or private aquariums and aquaculture facilities and causing mass mortalities of commercially-important species | Direct economic impacts on the aquarium and aquaculture industries and potential social impacts resulting from mass mortalities. *S. agalactiae* has already caused two major disease epizootics in stingrays in a commercial Aquarium | Major x Unlikely/possible = Medium/High | • Development of an advisory notice to communicate to the aquaculture and aquarium industries the potential for disease transmission with translocation of identified high-risk species.<br>• Review risks around translocation of high-risk species in the GBRMPA *'POSITION STATEMENT: Great Barrier Reef Marine Park Authority Position Statement on the translocation of species in the Great Barrier Reef Marine Park'*. | Minor x Unlikely/possible = Low |
| Impact on Marine Industries and Aquaculture | Risks to dive-based tourism if large Queensland Grouper are being removed from high-visitation dive sites. | Queensland grouper are an iconic fish at some high-visitation dive sites and loss of these individuals could result in loss of income, decrease in the social values of the GBR; reduced attraction of these sites for tourism and potential political risk. | Moderate x Unlikely = Medium. | • Use of 'Eye on the Reef' sightings network to identify whether Queensland Grouper are absent from recorded locations.<br>• Use tourism operators to report (and collect for sampling) Queensland Grouper carcasses.<br>• Inform relevant stakeholders and industry using standard and targeted communications, i.e. 'From the Deck' updates. | Unsure because suggested management controls are about increasing understanding and awareness, not directly related to reducing risk. |
| Impact on Marine Industries and Aquaculture | Risks to the commercial and recreational fishing industries should *S. agalactiae* cause mortalities of important fin-fish species. | *S. agalactiae* has been recorded causing mortalities of javelin fish in Trinity Inlet, Cairns, and other species like bluefish (= Australian Tailor) in Chesapeake Bay, USA (Baya *et al*. 1990). Lethal and non-lethal infections could impact on the economic viability of the inshore net fishery, fishing | Major x possible = High | • Support for Dr Bowater's FRDC project to improve knowledge and understanding, especially the field-based component to determine whether other fish species are carriers of *S. agalactiae*.<br>• Maintain reporting network and on-going response to | Unsure because suggested management controls are about increasing understanding and awareness, not directly related to reducing risk. |

| | | | | | |
|---|---|---|---|---|---|
| | | charters and social impacts on commercial and recreational fishers. | | Queensland Grouper carcasses<br>• Distribute the outcomes of the risk assessment process to Fisheries Queensland, Biosecurity Queensland, DERM and GBRMPA and revisit strategy and incidence response plan.<br>• Specific communications with the Queensland Seafood Industry Association important to ensure commercial fishers are made aware of the situation and to form an important 'in-field' notification network.<br>• Inform relevant stakeholders and industry using standard and targeted communications. | |
| Direct impact of *S. agalactiae* on Queensland Grouper and other species and subsequent impacts on the ecosystem. | Possible risk of the infection spreading along the coast, both North-West and South into other locations, such as the Gulf of Carpentaria, and south-east Queensland and NSW where Queensland grouper and other fish/ray species are found. | Mortalities of Queensland Grouper now recorded in the Gulf of Carpentaria, the Daintree River Yeppoon, and in the Brisbane River, with confirmed deaths from *S. agalactiae* now recorded in the Gulf of Carpentaria and as far south as Townsville. A spread of the disease would have impacts on these species directly; there could be flow-on effects to the ecosystem and subsequent impacts on the social amenity of the GBR. | Moderate x Possible/likely = Medium/High | • Support for Dr Bowater's FRDC project to improve knowledge and understanding<br>• Expand the geographical scope of Dr Bowater's field-based sampling to include outlier areas.<br>• Maintain reporting network and on-going response to Queensland Grouper carcasses.<br>• Distribute the outcomes of the risk assessment process to Fisheries Queensland, Biosecurity Queensland, DERM and GBRMPA and revisit strategy and incidence response plan.<br>• Inform relevant stakeholders and industry using standard and targeted communications.<br>• Better education and raising awareness of the issue. Link into wildlife disease database Taronga Zoo. | Unsure because suggested management controls are about increasing understanding and awareness, not directly related to reducing risk. |

| Direct impact of *S. agalactiae* on Queensland Grouper and other species and subsequent impacts on the ecosystem. | Direct impact on resident Queensland grouper populations, especially mortalities of large individuals. | Selective removal of large fish from inshore habitats could have flow-on effect to reduce the number of males in the population, as groupers change sex from female to a few dominant males. Little is known about the breeding cycle of this species, so mortalities of large individuals may impact on spawning if these are males. | Major x Likely = High | • Support for Dr Bowater's FRDC project to improve knowledge and understanding<br>• Maintain reporting network and on-going response to Queensland Grouper carcasses and amend sampling protocols to get data on the sex of each animal.<br>• Sampling of live animals to determine health status and | Unsure because suggested management controls are about increasing understanding and awareness, not directly related to reducing risk. |
|---|---|---|---|---|---|
| Direct impact of *S. agalactiae* on Queensland Grouper and other species and subsequent impacts on the ecosystem. | Ecosystem-level effects caused by the removal of apex predators (Queensland Grouper) from near shore habitats. | Removal of Queensland Grouper as apex predators in the near shore habitats could result in trophic cascades. | Minor/insignificant x possible = Low Risk | • Support for Dr Bowater's FRDC project to improve knowledge and understanding<br>• Maintain reporting network and on-going response to Queensland Grouper carcasses and amend sampling protocols to get data on the sex of each animal.<br>• Sampling of live animals to determine health status and | Unsure because suggested management controls are about increasing understanding and awareness, not directly related to reducing risk. |
| Direct impact of *S. agalactiae* on Queensland Grouper and other species and subsequent impacts on the ecosystem. | Potential disease transmission into marine mammals and other taxa that feed on infected prey species | There has been a confirmed death of a dolphin that consumed mullet that died from bacterial septicaemia caused by *S. agalactiae.* Potential for disease transmission into the three species of inshore dolphin and other species that might feed on fish infected with the bacterium. | Minor x Unlikely = Low Risk | • Support for the DERM marine stranding program and ensuring where possible that veterinarians conduct gross and histo-pathological examinations of dead dolphins.<br>• Better education and raising awareness of the issue. Communication via the DERM marine stranding hotline and raise awareness with veterinarians at those locations where necropsies are most likely to occur (The University of Queensland and James Cook University).<br>• Link into wildlife disease database Taronga Zoo. | Unsure because suggested management controls are about increasing understanding and awareness, not directly related to reducing risk. |
| Impacts on human health | Potential disease transmission to humans eating/handling fish or other species infected by *S. agalactiae.* | There is the potential for disease transmission if people eat undercooked fish infected with *S. agalactiae.* The bacterium is destroyed by cooking. There is the potential for people to develop tropical ulcers from exposure of abraded or cut skin to the bacterium, in fish handlers, aquaculturists, fishermen. | Minor x Possible = Low Risk | • Better education and raising awareness of the issue and educating fishers about the risks of eating infected fish.<br>• Better education and raising awareness about the potential risks of being exposed to *S. agalactiae* when handling infected fish. | |

# APPENDIX 11. PROPOSED ACTION PLAN FOR DISSEMINATING THE RESULTS OF THE QUEENSLAND GROUPER RISK ASSESSMENT

A workshop was conducted with a small group of scientists, DAFF veterinary pathologists, DERM scientists, and GBRMPA Marine Park Managers in 2010 to perform a risk assessment to:

1. Summarise and then rank (in order of impact) the current and potential hazards posed by the bacterium *S. agalactiae* to Queensland grouper (*Epinephelus lanceolatus*) and other fish species along the Queensland coastline; and
2. To identify and develop appropriate and sensible management responses to each hazard to reduce the risk.

The risk assessment indicated that high risks exist for *S. agalactiae* to continue to cause lethal disease in Queensland grouper and for the bacterium to spread along the Queensland coast to infect other Queensland grouper in near shore habitats. The risks of the bacterium causing lethal disease in other fin-fish species in the near shore habitats and this leading to flow-on impacts for commercial and recreational fisheries and the ecosystem was also considered to be 'high'. In addition, the other area where the risk was considered to be 'high' was for the bacterium to enter (via translocation of an infected fish) and cause significant mortalities and epizootics in marine aquaria and commercial marine aquaculture facilities.

It is now necessary to ensure the results from the risk assessment and the key actions to minimise risk are identified and tasked to relevant agencies and industry groups. This proposed action plan is the initial means of capturing that information and is intended to have two phases, the initial one involving providing the results from the risk assessment to the lead agencies, the second phase, following general agreement from the lead agencies, is to identify those tasks required to communicate this information to industry and other stakeholders

**Table 1 Proposed tasks and actions**

| Identified task | Suggested method for communicating results | Suggested lead agency |
|---|---|---|
| **Phase One:** | | |
| Dissemination of the risk assessment to appropriate agencies | Formal letter from Chair of the GBRMPA to:<br>• DEEDI (Chief Biosecurity Officer, Biosecurity Queensland)<br>• DEEDI (Managing Director, Fisheries Queensland)<br>• DERM (Dr Julia Playford, Director Water Quality and Aquatic Ecosystem Health) | GBRMPA to action |

| | | |
|---|---|---|
| | • Cairns Regional Council | |
| **Phase Two:** | | |
| Raise public awareness about the risks of eating infected fish and how to minimise risk | • Fact sheet (and new media release?) | • BQ (in consultation with Queensland Health) |
| Raise public awareness to ensure dead and dying fish are reported so samples can be collected for microbiology and pathology analyses | • Fact sheet?<br>• Message on DERM's marine stranding webpage<br>• Use QSIA communication tools<br>• Communication messages through the Eye-on-the-Reef network | • Factsheet (BQ)<br>• Message on DERM webpage (DERM)<br>• Article in the QSIA magazine<br>• Targeted communication through the Eye-on-the-Reef network |
| Advising the commercial fishing industry about the risks posed by *S. agalactiae* and the potential to impact species of commercial interest in the wild | • Develop a standard factsheet with QSIA with the request that the relevant information be forwarded to industry members | GBRMPA, BQ and FQ |
| Advising the marine aquarium supply industry of the potential risks of translocating fish infected with *S. agalactiae* into marine aquaria | • Develop an advisory notice with QSIA and ProVision Reef for distribution to industry members to make them aware of the potential risks associated with distributing infected fish to marine aquaria | BQ and FQ |
| Advising the marine aquaculture industry of the potential risks of translocating fish infected with *S. agalactiae* into marine aquaculture facilities | • Develop an advisory notice with Queensland Aquaculture Industry Federation for distribution to industry members to make them aware of the potential risks associated with distributing infected fish to marine aquaculture facilities<br>• Highlight the potential link between broodstock infected with *S. agalactiae* originating from both the GBR and the Gulf of Carpentaria | BQ and FQ |

Abbreviations:
BQ – Biosecurity Queensland
DERM – Department of the Environment and Resource Management
FQ – Fisheries Queensland
QSIA – Queensland Seafood Industry Association

# APPENDIX 12. QUEENSLAND GROPER MORTALITY INVESTIGATION TASK FORCE FACT SHEET

## Queensland Groper Mortality Investigation Task Force

### Report sick or dying groper and finfish

June 2009

Fact sheet

## Groper fish deaths

Since October 2007, Queensland Primary Industries and Fisheries (QPIF) has received more than 50 reports of dead Queensland groper (*Epinephelus lanceolatus*) on beaches and in waterways between Cooktown and Cairns.

Reports have also been received from the Cardwell, Mackay, Mission Beach, Burdekin and Bowen areas.

Testing has found the bacterium, *Streptococcus agalactiae*, to have caused fatal blood-poisoning in some of these fish.

This is first time this bacteria has been found in sick fish in Australia. It has been linked to fish deaths in the Middle East, the United States and Thailand.

QPIF wants to know how widespread the bacteria is, how it is transmitted, if environmental factors are involved, and why Queensland groper in the coastal north Queensland region appear to be susceptible to the bacteria.

### Other marine finfish

Two sick grunter taken from Trinity Inlet in early 2009 were also found to be infected with the bacteria. Healthy grunters tested were found to be free from the bacteria.

QPIF has tested several fish species from Trinity Inlet this year and has found the bacteria in a single mullet and a catfish.

Fishers should not be alarmed as the bacteria are destroyed when fish is cooked.

***It is safe to enjoy fishing in the far north and to eat cooked fish from this area.***

Photo of Groper supplied by Great Barrier Reef Marine Park Authority

### We need your help

Recreational fishers and beach-goers can assist with our investigation.

If you see a dead Queensland groper or any other dead fish species in the water or washed up on a beach or in mangroves, please contact QPIF immediately on **13 25 23**.

Early reporting is vital. QPIF fisheries scientists need to access fresh carcasses quickly to test the best possible samples.

The best samples are those taken from sick or dying fish. Recreational boaters and fishers are asked to promptly report any fish they see showing symptoms of sickness.

### Symptoms of a dying groper

- Underweight and apparent difficulty in feeding
- Lighter in colour than a healthy groper
- Skin lesions
- Cloudy or bulging eyes
- Weak swimming or swimming near the surface.

### Do not touch dead fish

If you find a dead fish, do not touch it.

If you do touch a dead fish, you are advised to wash your hands as soon as possible.

Queensland Government

**Department of Agriculture, Fisheries and Forestry**
**13 25 23**
**www.fisheries.qld.gov.au**