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Campylobacter genotypes in chickens

National and regional influences

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Campylobacter genotypes in chickens – national and regional influences

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Foreword

Food-borne illness caused by *Campylobacter* continues to be the most frequently reported notifiable infection in humans in Australia, with annual rates about double those for *Salmonella* spp.

Many studies in recent years have shown that there are multiple non-poultry potential sources for the transmission of *Campylobacter* into the human population. But it is still widely recognised that poultry is a major source of transmission for humans.

The objective of this project was to validate and apply a DNA-based typing scheme to understand the distribution of genotypes of *Campylobacter jejuni* (*C. jejuni)* across:

- a national company
- a production region featuring multiple companies
- several years in two production regions.

This publication describes the validation and application of Multilocus Sequence Typing (MLST) Single nucleotide polymorphism (SNP) High-Resolution Melt (HRM) typing to more than 650 *C. jejuni* isolates.

These isolates were collected predominantly from poultry at slaughter from a national survey of one company and from two regional surveys that encompassed multiple companies in a single production region. A comparison of genotypes in the two regional areas across time was also completed.

A key finding from this project was that MLST SNP HRM typing is a convenient first tool for screening *Campylobacter* isolates.

It has potential to contribute to a better understanding of the incidence and distribution of genotypes of *C. jejuni* throughout the Australian chicken meat industry and the potential influences of region, company and time on the presence of genotypes. This is significant for the chicken industry, food safety professionals and the industry research body.

This project was funded from industry revenue which is matched by funds provided by the Australian government.

This report is an addition to RIRDC's diverse range of over 2000 research publications and it forms part of our Chicken Meat R&D program, which aims to support increased sustainability and profitability in the chicken meat industry.

Most of RIRDC's publications are available for viewing, free downloading or purchasing online at www.rirdc.gov.au. Purchases can also be made by phoning 1300 634 313.

Craig Burns
Managing Director
Rural Industries Research and Development Corporation

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The output of this project is due largely to the high level technical skills and dedicated work of Jan-Maree Hewitson. I would also like to thank Jan-Maree for taking on the role of project leader during the first year of the project. Her input and enthusiasm is greatly appreciated, as is her friendship.

We would like to thank Dr Amy Jennison (Qld Health), Dr Sharon Chen (NSW Health) and Dr Tom Olma (NSW Health) for their willingness to contribute *Campylobacter* isolates to the project.

Abbreviations

CRISPR	Clustered, regularly interspaced short palindromic repeat sequences
DAFF	Department of Agriculture, Fisheries and Forestry
DNA	Deoxyribonucleic acid
D	Simpson's index of diversity
HRM	High-Resolution Melt
MLST	Multilocus Sequence Typing
NQ	North Queensland
PCR	Polymerase chain reaction
PFGE	Pulsed field gel electrophoresis
RFLP	Restriction fragment length polymorphism
RIRDC	Rural Industries Research and Development Corporation
SEQ	South East Queensland
SNP	Single nucleotide polymorphism
ST	Sequence type
SVR	Short Variable Region

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Executive Summary

What the report is about

This report describes the validation and application of a DNA-based typing scheme to more than 650 *Campylobacter* isolates, originating predominantly from poultry sourced across Australia.

Food-borne illness caused by *Campylobacter* continues to be the most frequently reported notifiable infection in humans in Australia, with annual rates of infection about double those for *Salmonella spp*.

Who is the report targeted at?

This report is targeted at Australian chicken meat processing companies, food safety professionals and food safety regulators.

Where are the relevant industries located in Australia?

The Australian poultry industry is located predominantly on the eastern seaboard in New South Wales (NSW), Victoria (Vic) and Queensland (Qld) and operators are generally established near capital cities.

In 2011, about one million tonnes of chicken meat was produced in Australia and the domestic industry employs about 40,000 people.

Meat chickens are grown on about 820 farms across the country and the gross value of production for the Australian poultry meat industry in 2005-06 was $1.408 billion.

Background

Raw or under-cooked poultry products are generally accepted as the major source of human *Campylobacter* infections. But several studies in recent years have challenged this idea.

The introduction of Multilocus Sequence Typing (MLST) in 2001 and subsequent widespread adoption of this technique as the new 'gold standard' for typing *Campylobacter* has provided researchers and food safety professionals with a tool for the reproducible and portable classification of *Campylobacter* isolates that is comparable around the world via an online database.

Numerous international studies investigating the source of *Campylobacter* infections in humans have used MLST to confirm that while poultry is not the sole source, it is one of the major sources in many countries.

The high cost of MLST has been overcome by examining Single nucleotide polymorphisms (SNPs), identified by computer software as being almost as informative as complete MLST.

This new technology uses a combination of kinetic Polymerase chain reaction (PCR) and interrogative data analysis that provides the power of conventional MLST, but at a lower cost and in a more rapid response time. In recent years, the cost has been further reduced with the introduction of High-Resolution Melt (HRM) analysis. This new technology is called MLST SNP HRM typing.

Aims/objectives

- Understand variation in *Campylobacter* genotypes at a national level
- Understand variation in *Campylobacter* genotypes at a regional level
- Understand variation in *Campylobacter* genotypes across multiple years.

Methods used

In the initial phase of this project, MLST SNP HRM typing was applied to a well characterised set of 62 *C. jejuni* isolates that had been characterised in the previous project (PRJ-000605) by the following techniques:

- SNP typing – based on seven housekeeping genes derived from MLST
- PFGE typing – whole genome restriction and electrophoresis
- Binary typing – analysis of the presence/absence of nine binary genes
- CRISPR typing – analysis of repetitive short palindromic sequences
- *flaA* HRM typing – HRM analysis of the short variable region of the *flaA* gene.

For the remainder of the project, MLST SNP HRM typing was applied to 652 isolates. The isolates consisted of:

- 259 *C. jejuni* isolates from one national company operating in the states of Qld, NSW, Vic, South Australia (SA), Western Australia (WA) and Tasmania (Tas)
- 163 *C. jejuni* isolates from several companies operating in an intensive production region of NSW
- 143 *C. jejuni* isolates from several companies operating in an intensive production region of south east Qld
- 42 *C. jejuni* isolates from human cases of campylobacteriosis in NSW
- 45 *C. jejuni* isolates from poultry products associated with a large outbreak of human campylobacteriosis in north Qld from one company.

Results/key findings

In the initial phase of the study, MLST SNP HRM typing was applied to 62 *C. jejuni* isolates that were previously characterised by several different genotyping methods and for which there was extensive epidemiological information.

Even though the use of MLST SNP HRM slightly reduced the ability to distinguish between isolates, it is a useful method for genotyping *C. jejuni* isolates - providing a discriminatory power above the ideal value for a typing method.

Therefore, we suggest that MLST SNP HRM would be a good first tool for screening a large collection of isolates in an investigation. This could be followed by either PFGE or full MLST on a smaller subset of isolates if further clarification is required.

The advantage of HRM analysis is that it is a portable, robust and cost-effective typing system that can be performed relatively quickly on new generation real-time PCR machines.

In the second part of the study, MLST SNP HRM typing was applied to 652 *C. jejuni* isolates collected during a national survey from one company and from two regional surveys in NSW and Qld from multiple companies in a production region in each state.

There was also a study to investigate isolates in a specific region across multiple years. In NSW, isolates were compared from years one and two of the project and in Qld, isolates were compared from years one and three.

Results showed no one dominant genotype was found in samples from the national company studied.

A small number of genotypes were common across flocks in different states, suggesting possible common environmental sources of *C. jejuni*.

A unique finding in the Qld regional survey was a marked dominance of one genotype in one company, with 78% of isolates being a single genotype.

There are shared genotypes across different companies in a geographical region, suggesting shared environmental sources.

Some genotypes can persist across time within a region, while others do not. One particular genotype, unresolved - or NT-1 (possibly *C. Coli*) - is an abundant genotype in a NSW region that was detected across companies and time. This genotype has also emerged in a Qld region, but only in one company and is absent in other companies operating in the same region. Free range flocks share essentially the same genotypes as those found in conventionally raised flocks.

Overall, MLST SNP HRM typing has been shown to be a convenient first tool for screening *Campylobacter* isolates, with comparable resolving power to other methods. The technique is user-friendly, relatively cheap and lends itself to robotics for sample preparation and assay set up.

Implications for relevant stakeholders

- there is evidence that a company can have a dominant genotype of *C. jejuni* that persists for at least one year and across multiple farms
- the domination of a genotype is not the normal situation, as all other companies (three in Qld and four in NSW) did not show this effect
- there is evidence of a regional influence that results in companies in a region showing shared genotypes of *C. jejuni* in poultry at slaughter
- there is mixed evidence that some genotypes persist across multiple years and other genotypes do not
- free range flocks show a similar range of genotypes of *C. jejuni* as those detected in conventional flocks. This suggests there is no unique risk, in terms of genotypes associated with *C. jejuni* colonisation, associated with free range production systems
- there is dominance in NSW of a novel genotype (possibly *C. coli*) across companies in one production region. The genotype has persisted in that region and has now also emerged in one company in Qld.

Recommendations

Several recommendations for food safety professionals, regulators and the industry research body have resulted from this study:

- MLST SNP HRM should be considered as a front-line method when typing *C. jejuni*
- a single genotype can become dominant within a company, therefore, consideration should be given to regular genotype monitoring to detect such occurrences
- further studies that enable a confident identification (at the species level) and understanding of the public health implications of the unresolved, or NT-1 genotype, now dominant in NSW and emerging in Qld, is required.

Introduction

In many developing countries, such as the USA, the UK and Australia, *C. jejuni* is the most frequently reported cause of food-borne gastrointestinal disease. The highest age-specific rate of infection in Australia is in zero to four-year-old children (188 cases per 100,000 head of population; Anon. 2001). The incidence in developing countries can be higher by several orders of magnitude, particularly in the very young (40,000 per 100,000 in children under five-years-old; Oberhelman and Taylor 2000).

Human infections caused by *C. jejuni* and the closely related *Campylobacter coli* primarily involve acute diarrhoea of varying severity, which may last from two to 10 days. Fever, headache and dizziness may precede the onset of diarrhoea (Skirrow and Blaser 2000). The severity of the accompanying abdominal pain, which may be misdiagnosed as acute appendicitis, is a distinguishing feature of *Campylobacter* enteritis (Skirrow and Blaser 2000).

Large outbreaks of *Campylobacter* are not common and the vast majority of *Campylobacter* infections occur as sporadic cases. This is probably why *Campylobacter* has escaped the headlines associated with outbreaks of food-borne pathogens such as *Escherichia coli* 0157 or *Salmonella ssp.* that have attracted major media attention. Also, few outbreaks are traced to a specific source - mainly due to the sporadic nature of the outbreaks (Corry and Atabay 2001). The low infectious dose for humans, about 500 organisms (Robinson 1981), means errors in food handling may result in human infection - even though campylobacters will not grow readily on foods at room temperature (Doyle and Jones 1992) and thorough cooking will eliminate campylobacters (Blankenship and Craven 1982).

The consumption of either contaminated water or unpasteurised milk is usually associated with common source outbreaks of campylobacteriosis when they occur (Skirrow and Blaser 2000). Contact with pets, especially puppies or kittens with *Campylobacter* diarrhoea, accounts for some sporadic cases (Skirrow 1991).

Raw or undercooked poultry products have been generally accepted as the major source of human *Campylobacter* infections (Oosterom *et al*. 1984; Deming *et al*. 1987). But, in several studies in recent years, the traditional acceptance of the primary role of chicken products in the transmission of *Campylobacter* into the human population has been challenged.

Studies using molecular typing techniques have indicated that a significant proportion of *Campylobacter* from humans were not of poultry origin (Koenraad *et al*. 1995; Hudson *et al*. 1999) and that some originated from cattle (On *et al*. 1998; Colles *et al*. 2003).

Molecular typing of *Campylobacter jejuni/coli*

A range of molecular typing methods for *C. jejuni* and *C. coli* have been described (Wassenaar and Newell 2000). It is beyond the scope of this introduction to provide a full description of the various molecular typing methods. Basic descriptions of the various techniques are provided in the review by Wassenaar and Newell (2000).

In the past, the commonly applied methods for the molecular typing of *C. jejuni/coli* were *fla*A (or *fla*B) gene PCR-based typing (with some use of sequencing, rather than typing) and PFGE (Wassenaar and Newell 2000). But, difficulties in the reproducibility and interpretation of results among laboratories has limited the ability of these techniques to become unified typing schemes by which *Campylobacter* isolates could be compared on a wider scale (Wassenaar and Newell 2000).

In recent years, MLST has become the new ‘gold standard’ for typing of *Campylobacter* and many other bacteria. MLST discriminates *C. jejuni* and *C. coli* isolates based on sequence variation in seven housekeeping genes (Maiden *et al*. 1998). MLST-based studies have provided insights into key

aspects of the *Campylobacter* population structure and epidemiology and enabled identification of host and geographical niches of genotypes (Mickan *et al*. 2007; Foley *et al*. 2009; Sheppard *et al*. 2009a; Strachan *et al*. 2009). But MLST is problematic for routine surveillance in small laboratories because of its multi-step nature, time consuming data analysis and significant equipment and on-going costs (Price *et al*. 2006b).

An alternative approach is the adaption of MLST to the more cost-effective and rapid detection technology of SNPs -, with these SNPs detected by such methods as real time PCR (Best *et al*. 2004). This method was used in our original SNP typing technique in the previous project (PRJ-000605) (Templeton 2009).

In PRJ-000605, SNP typing was successfully applied to a collection of more than 500 *Campylobacter* isolates originating from different host species (humans, chickens, dairy cattle, beef cattle, feedlot cattle, dogs and cats) to clarify the distribution of genotypes within these species. Results showed that some genotypes were associated with multiple host species and other genotypes were predominantly associated with limited host species. For example, SNP type 44 was a genotype found in humans, dogs and cats, SNP type five was associated with dairy cattle and SNP type 13 was associated with feedlot cattle (Templeton 2009).

Even this real time PCR SNP analysis (SNP tying) used in project PRJ-000605 had practical and cost limitations. The SNP typing approach involved interrogation of seven SNPs across seven different MLST genes using allele specific PCR reactions requiring 15 primer sets. This required 15 different PCR reactions in individual tubes to type one isolate - a time consuming and costly exercise.

As an emerging genotyping technology, HRM analysis is inherently attractive because it is single step, closed tube, cost effective and very robust (Merchant Patel, 2009). It has recently been shown that it is possible to discriminate multiple alleles in a single reaction (Seipp *et al*. 2009), thus making the potential combinatorial resolving power of genotyping procedures involving several HRM reactions very high.

Recently, a HRM-based variant of the real time PCR SNP analysis has been developed at the Menzies School of Health Research (Merchant Patel, 2009). This involves deriving a resolution optimised SNP set from the relevant MLST database and then defining small fragments that incorporate these SNPs and are suitable for HRM interrogation. The attractiveness of this approach is the additional SNPs in the fragments potentially provide extra resolving power - and the method has the inherent robustness of HRM.

Campylobacter and poultry

C. jejuni is part of the normal gut flora of many warm-blooded animals, including food production animals and pets (Franco 1988; Newell and Fearnley 2003). *C. jejuni* has fastidious growth requirements and appears to be adapted for growth in the avian gut (Manning *et al*. 2003). The optimum growth temperature for *C. jejuni* and *C. coli* is about 42ºC, which is the body temperature of a chicken (Shane 1992).

The primary site of colonisation in the chicken is in the lower gastrointestinal tract, especially the caeca (Beery *et al*. 1988). Levels of the organism in caecal contents can be as high as 10^6 to 10^7 per gram (Mead *et al*. 1995). During evisceration, campylobacters can spill over onto the carcass. Further cross-contamination can occur during the spin-chilling process. Prevalence at the retail level can be very high, with overseas studies reporting up to 98% of samples being *Campylobacter*-positive (Jacobs-Reitsma 2000).

It is rare to isolate campylobacters from chicks in the first two weeks of life (Jacobs-Reitsma *et al*. 1995; Evans and Sayers 2000). Once introduced, the organism spreads rapidly throughout the entire

flock (Evans 1992). Many studies have been undertaken around the world to determine the source of contamination on commercial poultry farms.

Horizontal transmission is generally considered the most significant cause of *C. jejuni* infection in broiler flocks (Newell and Fearnley 2003). Campylobacters are ubiquitous in the environment and could be readily carried into the sheds by several modes, including human activity associated with routine flock management (Newell and Fearnley 2003). A high priority in reducing *Campylobacter* levels in chickens is still good biosecurity.

MLST typing of strains provides a way for 'environmental contamination' to be more accurately defined and attributed to agricultural or wildlife sources (Colles and Maiden 2012).

As noted above, poultry is a major - but not the only - source of *Campylobacter* infections in humans. As an example, molecular typing studies in New Zealand during the past six to eight years have shown direct connections between poultry meat and human campylobacteriosis. Mullner *et al*. (2010a) showed there was an overlap of 21 out of 51 genotypes in human and poultry isolates, with these 21 genotypes accounting for 85.5% of the human cases. This study also showed the dominant human sequence type in New Zealand was found almost exclusively in isolates from one poultry supplier (Mullner *et al*. 2010a). These New Zealand findings have practical implications for focused control programs that aim to reduce human campylobacteriosis.

It is possible in Australia that certain genotypes of *C. jejuni* are common within a company and/or common within a production region. The relative distribution of genotypes within a company and across companies within a region is currently not understood in this country. If certain genotypes are common to a region/company and are also dominant in the human population, then focused control programs are clearly an option.

Overall, there is a clear and evident need for a study that seeks to provide basic knowledge about the distribution of genotypes of *C. jejuni* across a company and across a production region that features multiple companies.

Objectives

- Understand variation in *Campylobacter* genotypes at a national level
- Understand variation in *Campylobacter* genotypes at a regional level
- Understand variation in *Campylobacter* genotypes over time.

Methodology

Bacteriology

Caecal samples

Caecal samples collected in the abattoir were held on ice during transport to the laboratory. All caecal samples were streaked directly onto *Campylobacter* Blood-Free Selective Agar Base (Oxoid CM0739, Oxoid, Melbourne) containing CCDA Selective Supplement (Oxoid SR0155E). Samples were incubated at 42°C for 24-48 hours in a tri-gas incubator in an atmosphere of 85% N_2, 10% CO_2 and 5% O_2. On the basis of colony morphology, agar plates were recorded as having negative (non-*Campylobacter*) growth or suspect (*Campylobacter)* growth.

Isolates showing suspect colony morphology were examined by phase contrast microscopy for typical *Campylobacter* motility. Three presumptive *Campylobacte*r isolates (labelled a, b and c) from each positive caecal sample were subcultured to Sheep Blood Agar (BBL Blood Agar Base, Becton Dickinson with 5% sheep blood) and incubated as above. Each isolate was subjected to two single colony subcultures for purification prior to storage.

Characterisation and storage of isolates

All presumptive *Campylobacter* isolates were identified to species level using real-time PCR detection of the *mapA* (for *C. jejuni*) or the *ceuE* (for *C. coli*) genes using a modification of the original methods (Stucki *et al*. 1995; Gonzalez *et al*. 1997) as described by Price *et al*. (2006a).

Isolates were stored as viable cultures at -70ºC in Tryptone Soya Broth (Oxoid CM0129) with 15% glycerol. As well, purified DNA was prepared using PrepMan Ultra Sample Preparation Reagent (Applied Biosystems) from the 'a' sample only and the extract stored at -20ºC.

Molecular typing

MLST SNP HRM typing

The identification of DNA fragments optimised for HRM-based genotyping was carried out using a two-step, bioinformatics-based analysis of the *C. jejuni*/*coli* MLST database. The process was designed to yield a small set of fragments from the MLST database which, when subjected to HRM analysis, would provide a typing method with high resolving power.

The first step involved identification of highly resolving SNPs. The second step involved the design of HRM fragments ensuring the inclusion of the highly resolving SNPs.

Identification of highly resolving SNPs from the MLST database was carried out using 'Minimum SNPs' (Robertson *et al*. 2004). The inputs for the Minimum SNPs software were the alleles and corresponding STs from the *C. jejuni* MLST database (http://pubmlst.org/campylobacter/). The discriminatory power of the MLST-SNPs was calculated by 'Minimum SNPs' using the Simpson's Index of Diversity (*D*).

MLST-SNPs identified by this process guide the identification of a set of fragments useful for HRM analysis, such that each fragment contains a SNP that was part of a resolution optimised SNP set. Although a number of high *D*-SNPs are identified, the sites containing the SNPs may not be

conducive for primer design. Functions within 'Minimum SNPs' were used to identify a small set of SNPs that provided high resolution and facilitated the design of a viable and robust PCR assay.

The second step of this process involved identification of fragments that include the selected SNPs and are suitable for HRM analysis. The identification of HRM fragments was driven by the use of a new computer program called HRMType ®, which was developed by Dr Steven Tong at the Menzies School of Health Research. The premise behind the method used was that change in the %G+C content of a fragment will result in a change in the HRM profile of that fragment. Thus, sequence variants of the HRM fragment differing in their %G+C content could be referred to as HRM alleles of that fragment.

Real-time PCR and HRM

Real-time PCR and HRM were performed on the Corbett RotorGene-6000 (Corbett Research, Sydney, Australia). DNA was amplified using 5 µL of Platinum® SYBR® Green qPCR SuperMix-UDG, 2 µL of template, 5 pmol of the forward and reverse primers and the final volume was made up to 10 µL with water.

Following real-time PCR amplification, the following HRM protocol was used: 95°C for two minutes, 95°C for one second and 61°C for 30 seconds for 40 cycles, 72°C hold for one minute and 50°C hold for 20 seconds, followed by HRM performed between 67°C and 80°C with 0.1°C increments.

The RotorGene software (version 1_7_87) was used for data analyses.

DNA Sequencing

MLST determination was performed according to Dingle *et al.* (2001).

Prior to sequencing, PCR products were purified using ExoSAPIT (VWR, Brisbane, Australia) according to the manufacturer's instructions. Between 10 and 40 ng of template DNA was mixed with relevant sequencing primer at a final concentration of 9.6 pmol in a 12 µL reaction. Sequencing was carried out at the Australian Genomic Research Facility, Brisbane, using a protocol of 96ºC for 1 min, 96ºC for 10 secs, 50ºC for 5 secs and 60ºC for 4 mins on the AB3730SL platform. Sequencing results were analysed using Sequencher v4.8 (Generesearch, Arundel, Australia) software.

Chapter 1: Validation of MLST SNP HRM

Introduction

The MLST SNP HRM methodology was originally developed by Dr Shreema Merchant-Patel and colleagues at the Menzies Institute in Darwin, but was not published.

The method is a HRM-based variant of the original SNP typing method described by Price *et al*. (2006a) and was used in the previous project (PRJ-000605). In contrast to the original method, where a single nucleotide at a particular position is determined, this method uses HRM analysis to interrogate the SNP as well as the sequence either side of the identified SNP. This has the benefit that these short DNA fragments may contain additional SNPs that can possibly provide additional resolving power.

HRM is based on accurate monitoring of the reduction in fluorescence as a PCR product - stained with a double-strand-specific fluorescent dye - is heated through its melting temperature (T_m). In contrast to traditional melting analysis, the information in HRM analysis is contained in the shape of the melting curve - rather than just the calculated T_m - so HRM analysis may be considered a form of spectroscopy. HRM analysis is single step and closed tube because the amplification and melting can be run as a single protocol on a real-time PCR device (Merchant-Patel 2009).

To validate this new method, a study was conducted on the carefully selected subset of *Campylobacter* isolates used in the previous project (PRJ-000605) to assess different typing techniques.

Material and Methods

All isolates were obtained from chicken faecal samples, except for one *C. jejuni* isolate that was obtained from darkling beetle larvae collected during epidemiological studies based in SEQ as part of previous RIRDC-funded projects DAQ-245A (Miflin 2001) and DAQ-282A (Templeton and Miflin 2005). The isolates were shown to be *C. jejuni* by conventional PCR (Linton *et al*. 1997) and real-time PCR detection of *mapA* region (Price *et al*. 2006a).

Molecular Typing

All isolates were subjected to MLST SNP HRM typing as described earlier in the Methodologies sub-section titled ‘MLST SNP HRM typing’.

C. jejuni isolates

The following groups of isolates were used to evaluate the MLST SNP HRM method:

- Group 1 – a collection of 32 isolates, each representing a different *flaA* RFLP type
- Group 2 – consisted of three groups of 10 isolates with the same *flaA* RFLP type collected on the same day from one farm within one shed (*flaA* types I, VIII and XXVI).

In the previous project (PRJ-000605) both groups were subjected to the following genotyping techniques:

- SNP typing – based on seven housekeeping genes derived from MLST

- PFGE typing – whole genome restriction and electrophoresis
- Binary typing – analysis of the presence/absence of nine binary genes
- CRISPR typing – analysis of repetitive short palindromic sequences
- *flaA* HRM typing – HRM analysis of the short variable region of *flaA* gene.

Statistical Analysis

The Simpson's Index of Diversity (*D*) was used to assess the ability of a particular typing technique to discriminate within a group of isolates (Hunter and Gaston 1988).

Results

An example of typical MLST SNP HRM curves for each of the MLST regions is presented in Figure 1.1. This shows the HRM curves of 20 isolates across the six regions incorporated in the MLST SNP HRM typing method. For each region, new isolates were compared with known controls to determine the curve profile of each new isolate. For example, in the *pgm* region, there were 20 new isolates where two were Curve 17; 16 were Curve 19; and two were Curve 20.

The build-up of these curves for each of the regions determines the MLST SNP HRM type for each isolate. For example, an isolate with a MLST SNP HRM type of 206 has a profile made up of curves 19, 6, 13, 7, 24 and 19 (circled in red on Figure 1) for the regions *pgm*, *asp*, *gln*, *glt*, *unc* and *glt2* respectively. All the MLST SNP HRM types are determined by a key developed from the MLST database for *Campylobacter*.

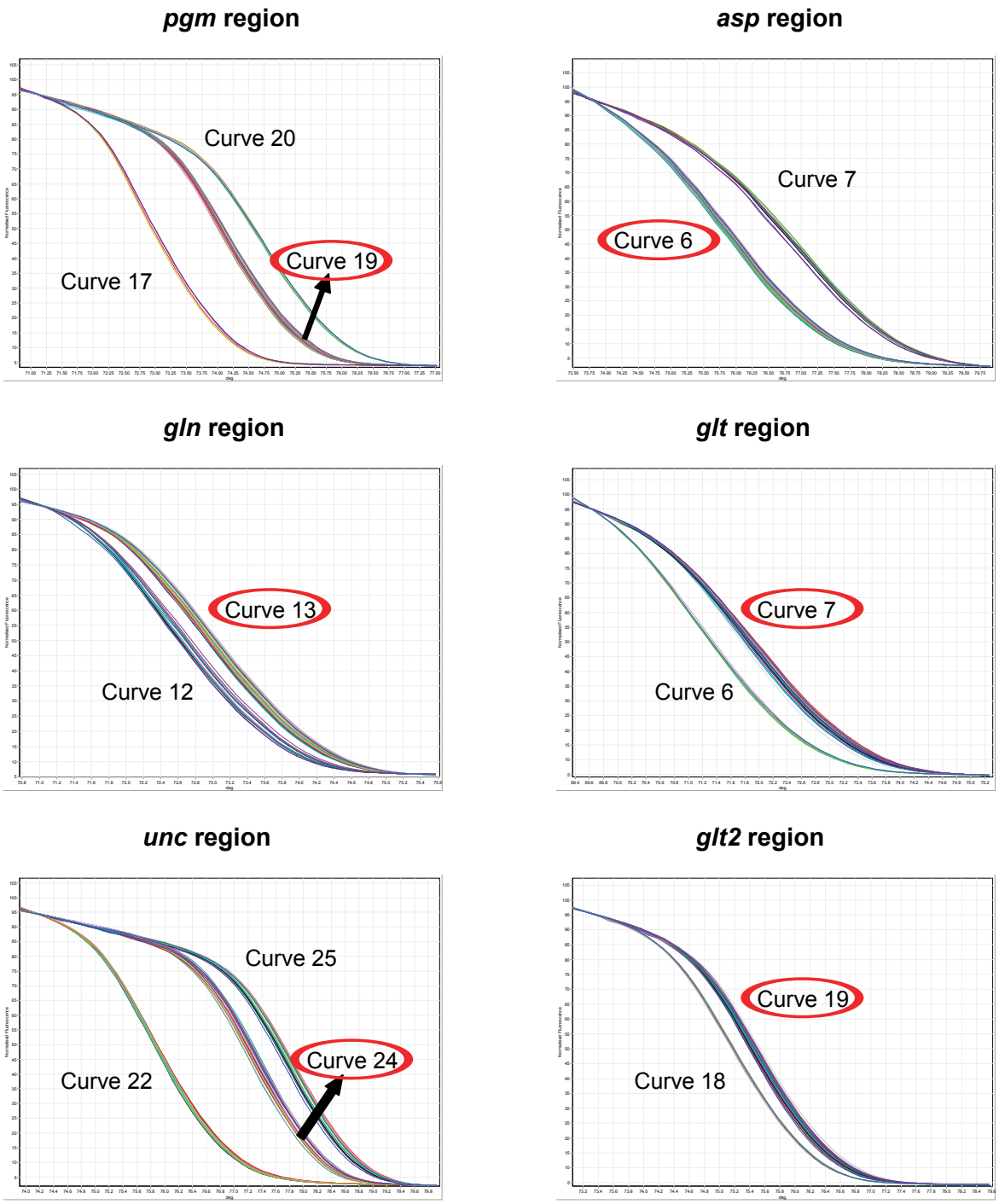

Figure 1.1 MLST SNP HRM curves for 20 isolates analysed across six MLST regions to determine the MLST SNP HRM type for each isolate. For example, MLST SNP HRM type 206 has a profile made up of curves 19, 6, 13, 7, 24 and 19 (circled in red) for the regions pgm, asp, gln, glt, unc and glt2 respectively.

The results from the MLST SNP HRM typing method for Group 1 *flaA* master types are presented in the shaded area in Table 1.1 below. This compares the results of MLST SNP HRM with results from other genotyping techniques used in the previous project (PRJ-000605). The isolates were selected to represent 32 distinct isolates based on *flaA* RFLP typing.

The MLST SNP HRM typing resolved the isolates into 17 distinct MLST SNP HRM types, with 10 MLST SNP HRM types having multiple isolates. In all cases, the isolates within a multi-isolate MLST SNP HRM type were differentiated from each other by *flaA* RFLP and by at least one other typing technique for each distinct group.

The results from the MLST SNP HRM typing method for Group 2, the collection of 30 isolates with three subsets of 10 isolates which are epidemiologically linked, are presented in Table 1.2 below. This compares the results of MLST SNP HRM with results from other genotyping techniques used in the previous project (PRJ-000605). The MLST SNP HRM typing method resolved the isolates into the three distinct subsets and there was no variation within each group.

The results for the statistical analysis of the five different typing techniques, the current MLST SNP HRM and the four techniques previously used, for Group 1 isolates, are presented in Table 1.3 below. The Group 2 isolates were used to assess the ability of the methods to hold together isolates with known epidemiological links - so the D-value for this group was not calculated.

Table 1.1 List of 32 C. jejuni flaA master types indicating origina, epidemiological information and additional genotyping results using SNP, PFGE, Binary, CRISPR and flaA HRM

Study Code	Farm Code	Date Collected	Company	*flaA* RFLP Type[b]	SNP Type[c] PRJ-000605	MLST SNP HRM Type	PFGE Type[c]	Binary Type[c]	CRISPR Type[c]	*flaA* HRM Type[c]
C1276	#84	Dec 2000	A	XXVI	1	87	23	1	1	33
C915	#7	Dec 1999	B	VII	1	87	23	2	1	24
C838	#23	Dec 1999	A	I	2	117	30a	16	2	1
C1208	#23	Dec 1999	A	II	3	289	32	2	1	35
C1274	#97	Nov 1999	C	XXV	4	206	1a	40	37	3
C775	#23	Oct 1999	A	XX	5	206	14	21	1	37
C1266	#40	Jan 2001	C	XVII	6	34	42	45	33	8
C1282	#40	Jan 2001	C	XXII	6	34	43	46	7	18
C674	#55	May 1999	B	IX	6	34	47	17	2	22
N15	#34	Oct 2000	B	LIII	6	34	42	17	1	32
C654	#9	May 1999	C	X	11	284	34	16	26	20
C858	#23	Dec 1999	A	VIII	11	284	36	30	25	23
C1212	#6	May 2000	B	V	12	310	18	14	23	7
C1275	#98	Dec 1999	C	XXVII	12	310	15	49	38	4
C1273	#99	Oct 1999	A	XXIV	13	290	20	41	36	17
C660	#54	May 1999	A	XI	14	309	54	15	27	21
C1303	#91	Jul 2003	A	LXI	15	309	40	15	22	21
C338	#14	Jan 1999	B	XIV	15	310	41	16	39	6
C1271	#100	Oct 1999	A	XXI	18	87	51	31	34	44
C541	#33	Mar 1999	A	XVI	21	289	25	31	7	36
C1270	#100	Oct 1999	A	XXIII	22	270	45	17	35	16
C1304	#91	Jul 2003	A	LVI	22	270	44	31	21	41
C627	#51	Apr 1999	C	XII	22	277	19	37	28	45

Study Code	Farm Code	Date Collected	Company	*flaA* RFLP Type[b]	SNP Type[c] PRJ-000605	MLST SNP HRM Type	PFGE Type[c]	Binary Type[c]	CRISPR Type[c]	*flaA* HRM Type[c]
C1209	#23	Jan 2000	A	III	28	208	11	42	19	10
C1272	#100	Oct 1999	A	XIX	28	208	12	43	30	40
C1211	#101	Mar 2000	B	IV	29	184	46	17	3	13
C350	#15	Jan 1999	A	XV	29	271	13	44	32	5
C576	#40	Mar 1999	C	XIII	31	189	24	7	29	9
C1301	#91	May 2003	A	XLVII	32	95	50	20	31	25
C1302	#91	Jul 2003	A	XLVIII	33	83	48	17	8	27
C1269	#100	Oct 1999	A	XVIII	35	277	17	18	10	15
C1210	#23	Apr 2000	A	VI	38	95	38	47	24	43

[a] All strains were obtained from chicken faecal samples with the exception that isolate C1302 was obtained from darkling beetle larvae.
[b] As described by Miflin *et al*. (2001) and Templeton and Miflin (2005).
[c] SNP types, PFGE types, Binary types, CRISPR types and *flaA* HRM types are allocated as described in the previous project report (PRJ-000605).

Table 1.2 List of 30 C. jejuni isolatesa consisting of three groups of 10 isolates with the same flaA RFLP type collected on the same day from one farm within one shed

Study Code	Source Code	Company	*flaA* RFLP types[b]	SNP Type[c] PRJ-000605	MLST SNP HRM Type	PFGE Type[c]	Binary Type[c]	CRISPR Type[c]	*flaA* HRM Type[c]
A529	Cycle 2, Shed 3, Day 37	A	I	2	117	30a	30	2	1
A531	Cycle 2, Shed 3, Day 37	A	I	2	117	30a	30	2	1
A533	Cycle 2, Shed 3, Day 37	A	I	2	117	30a	30	2	1
A535	Cycle 2, Shed 3, Day 37	A	I	2	117	30a	30	2	1
A537	Cycle 2, Shed 3, Day 37	A	I	2	117	30a	30	2	1
C1077	Cycle 2, Shed 3, Day 37	A	I	2	117	30a	30	2	1
C1078	Cycle 2, Shed 3, Day 37	A	I	2	117	30a	30	2	1
C1079	Cycle 2, Shed 3, Day 37	A	I	2	117	30a	30	2	1
C1080	Cycle 2, Shed 3, Day 37	A	I	2	117	30a	30	2	1
C1081	Cycle 2, Shed 3, Day 37	A	I	2	117	30a	30	2	1
L131	Cycle 2, Shed 6, Day 28	C	VIII	5	206	2	21	3	23
L132	Cycle 2, Shed 6, Day 28	C	VIII	5	206	2	21	3	23
L133	Cycle 2, Shed 6, Day 28	C	VIII	5	206	2	21	3	23
L134	Cycle 2, Shed 6, Day 28	C	VIII	5	206	2	21	3	23
L136	Cycle 2, Shed 6, Day 28	C	VIII	5	206	2	21	3	23
L137	Cycle 2, Shed 6, Day 28	C	VIII	5	206	2	21	3	23
L138	Cycle 2, Shed 6, Day 28	C	VIII	5	206	2	21	3	23
L140	Cycle 2, Shed 6, Day 28	C	VIII	5	206	2	21	3	23
L141	Cycle 2, Shed 6, Day 28	C	VIII	5	206	2	21	3	23
L142	Cycle 2, Shed 6, Day 28	C	VIII	5	206	2	21	3	23
N70	Cycle 2, Shed 1, Day 36	B	XXVI	1	87	25	1	1	33
N72	Cycle 2, Shed 1, Day 36	B	XXVI	1	87	25	1	1	33
N73	Cycle 2, Shed 1, Day 36	B	XXVI	1	87	25	1	1	33
N74	Cycle 2, Shed 1, Day 36	B	XXVI	1	87	25	1	1	33

Study Code	Source Code	Company	*flaA* RFLP types[b]	SNP Type[c] PRJ-000605	MLST SNP HRM Type	PFGE Type[c]	Binary Type[c]	CRISPR Type[c]	*flaA* HRM Type[c]
N75	Cycle 2, Shed 1, Day 36	B	XXVI	1	87	25	1	1	33
N78	Cycle 2, Shed 1, Day 36	B	XXVI	1	87	25	1	1	33
N79	Cycle 2, Shed 1, Day 36	B	XXVI	1	87	25	1	1	33
N81	Cycle 2, Shed 1, Day 36	B	XXVI	1	87	25	1	1	33
N82	Cycle 2, Shed 1, Day 36	B	XXVI	1	87	25	1	1	33
N84	Cycle 2, Shed 1, Day 36	B	XXVI	1	87	25	1	1	33

[a] All strains were obtained from chicken faecal samples.
[b] As described by Miflin *et al*. (2001) and Templeton and Miflin (2005).
[c] SNP types, PFGE types, Binary types, CRISPR types and *flaA* HRM types are allocated as described in the previous project report (PRJ-000605).

Table 1.3 Comparison of the Simpson's Index of Diversity (D) provided by the typing methods for Group 1.

Typing Method	No. of Types	*D*-value	CI (95%)
MLST SNP HRM	17	0.960	(0.940-0.980)
Binary	22	0.964	(0.932-0.996)
SNP	21	0.970	(0.948-0.992)
CRISPR	26	0.976	(0.944-1.000)
PFGE	30	0.996	(0.989-1.000)

Discussion

The aim of this study was to validate the ability of the new MLST SNP HRM typing method to differentiate chicken isolates of *C. jejuni*. The isolates used were originally selected on the basis of *flaA* RLFP typing.

The *Campylobacter* isolates were carefully selected from an extensive culture collection that was established during epidemiological studies of the SEQ chicken industry between 1999 and 2003. The strains were previously characterised by *flaA* RFLP typing and this, coupled with the detailed epidemiological information that accompanies each of the isolates, has created a unique collection to use to compare typing techniques.

An important feature of typing methods is their discriminatory power, that is, their ability to distinguish between unrelated isolates (Hunter and Gaston 1988). This power is determined by the number of types recognised and the relative frequency of those types. We have used the Simpson's Index of Diversity (*D*), as suggested by Hunter and Gaston (1988), to quantify this feature. It has been suggested that an index of 0.90 or higher is desirable if a typing system is to be useful (Hunter and Gaston 1988; Behringer *et al*. 2010).

Analysis of Group 1, a collection of 32 *C. jejuni flaA* RFLP type master isolates, showed that MLST SNP HRM gave comparable discrimination of the isolates as achieved by the other typing techniques. While MLST SNP HRM had the lowest overall *D*-value of the five methods examined, there are examples where it was able to distinguish between isolates grouped by other methods. As an example, CRISPR type 1 (the largest CRISPR type with five isolates) consisted of four MLST SNP HRM types (34, 87, 206 and 289). Likewise, there were times when MLST SNP HRM could not distinguish between isolates that were separated by other methods. For example, MLST SNP HRM type 87 with three isolates consisted of three CRISPR types, three Binary types and 2 PFGE types. Previous studies have shown that the use of multiple typing methods will often result in differing clusters or groups of isolates (Clark *et al*. 2011; Magnússon *et al*. 2011).

Another important feature of typing methods is the ability to specifically identify all related isolates (Ogle *et al*. 1987). The Group 2 isolates used in the study consisted of the three subsets of 10 isolates with the same *flaA* RFLP type, collected on a single day from one shed on one farm. Analysis of this group resulted in the isolates from each subset being held together in their respective subsets by the new MLST SNP HRM typing. This congruence between typing results and epidemiology (i.e. the true epidemiologically linked groups were clearly linked by MLST SNP HRM) is an important finding that increases confidence in applying MSLT SNP HRM.

But it appears use of MLST SNP HRM has slightly reduced the ability to distinguish between isolates, as shown by a *D*-value of 0.960 compared with *D*-values of 0.964 to 0.996 achieved by other methods. One explanation for this reduction, as suggested by Merchant-Patel (2009), is that we are no longer looking at just one SNP, but a region of DNA that contains the informative SNP. By including this

region of DNA before and after the SNP we may be including additional SNPs that can possibly provide additional resolving power. But it is possible that these additional SNPs cancel out the effect of other SNPs within the fragment, hence imparting similar HRM profiles to sequence variants. While not the highest *D*-value of the methods examined in this study, MLST SNP HRM did give a *D*-value of 0.96 above the 0.90 suggested by Hunter and Gaston (1988) and also above the 0.95 level suggested as ideal by Van Belkum *et al*. (2007).

In addition to absolute typing capacity, a range of other factors impact on the selection of a typing method. These factors include cost and the degree of difficulty in performing the technique and interpreting the results (Behringer *et al*. 2010). The SNP typing method used in the previous project (PRJ-000605) involved the interrogation of seven SNPs across seven different MLST genes using allele specific PCR reactions, which requires 15 primer sets. This necessitates 15 different PCR reactions in individual tubes to type one isolate.

In contrast, the current MLST SNP HRM typing method involves the interrogation of six MLST SNP regions across five genes using only six primer sets to amplify the regions of interest. By using HRM to analyse the PCR data, one reaction/tube is capable of discriminating multiple alleles. Therefore, MLST SNP HRM requires only six different PCR reactions in individual tubes to type one isolate – using less than half the reagents required by the previous method.

Overall, we have shown that MLST SNP HRM is a useful method for genotyping *C. jejuni* isolates - providing a *D*-value above the ideal level. We suggest that MLST SNP HRM would be a good first-line tool for screening a large collection of isolates in an investigation and then, if further clarification is required, to follow up with either PFGE or full MLST on a smaller subset of isolates. The advantage of HRM analysis is that it is a portable, robust and cost-effective typing system that can be performed on new generation real-time PCR machines.

Chapter 2: National Survey

Introduction

A study was conducted to use MLST SNP HRM typing to compare *C. jejuni* isolates from one company that operates across six States in Australia: Qld, NSW Vic, SA, WA and Tas. One sampling occasion was completed for Vic before the closure of the processing plant due to fire.

With RIRDC approval, samples from free range chickens in Qld were substituted for sampling rounds two, three and four - as well as samples from conventionally raised chickens from that state.

Sample collection in NSW and Qld focused on a defined region in each state to allow a study of genotypes over time within the project. Isolates collected in these defined regions will be compared to others collected in the same regions in years two and three of the project. The results of these multi-year studies will be presented in Chapter 5.

Collection of samples from NSW for the national survey focused on chickens reared in the Mangrove Mountain region. For this study, Mangrove Mountain has been defined as including farms north of the Hawkesbury River and south of Newcastle - with the majority of farms concentrated in a 20 kilometre radius of the locality of Mangrove Mountain. Collection of samples from Qld for the national survey focused on the Beaudesert and Redland Bay areas - defined as the South-East Brisbane region.

Material and Methods

Sample Collection

Whole chicken caeca were collected (as per Appendix 1) from randomly selected birds at slaughter by quality assurance staff in the relevant processing plants. Samples were collected on four occasions during a 12-month period. On each sampling occasion, four flocks from each processing facility in each state were sampled - except for Vic, which was only sampled in round one. Four flocks from Qld's free range flocks were also collected for sampling in rounds two, three and four. Only older birds (above 40 days of age) were sampled. The exception was for Tas, where - due to different production conditions - chickens are often processed at an earlier age. On each sampling occasion, five caeca from each flock were randomly selected. No first pick-up birds were sampled. Samples were shipped to the laboratory at 4ºC as soon as possible.

A total of 20 samples were collected from each of the states on each sampling occasion, plus 20 samples were collected from Qld free range flocks in sampling rounds two, three and four. A total of 120 samples were collected on each sampling occasion across Australia. This meant 480 samples were collected nationally for the 12 month period.

Sample Processing

Samples were processed on the day of arrival at the laboratory, as described earlier in the Methodologies sections 'Caecal Samples' and 'Characterisation and Storage of Isolates'.

Molecular Typing

All *C. jejuni* isolates were subjected to MLST SNP HRM typing, as described earlier in the Methodologies sub-section 'MLST SNP HRM typing'.

Results

Sample collection

Detailed results for the four individual sampling occasions are summarised in Tables 2.1, 2.2, 2.3 and 2.4 below. At each sampling occasion, five samples were collected from four different farms. The farms were labelled one, two, three and four for each sampling occasion - but these numbers do not carry over for each sampling occasion. For example, Qld farm one in sampling occasion one may not be the same farm as Qld farm one in sampling occasions two, three or four.

Table 2.1 Summary of samples collected for the first sampling occasion

Sampling Occasion 1	Samples		Isolates			
	positive	negative	*C. jejuni*[a]	*C. coli*[a]	Unresolved	# samples/farms
QLD	20	0	10 (1,2,4)	10 (1,2,3)	0	20 samples/4 farms
TAS	10	10	10 (1,3)	0	0	20 samples/4 farms
WA	15	5	15 (1,2,3)	0	0	20 samples/4 farms
SA	10	0	5 (2)	5 (1)	0	10 samples/2 farms[b]
NSW	20	0	11 (1,2,4)	0	9[c] (3,4)	20 samples/4 farms
VIC	18	2	11 (2,3,4)	7 (1,2)	0	20 samples/4 farms
QLD-FR[d]	5	0	5 (1)	0	0	5 samples/1 farm
TOTAL	98	17	67	22	9	

[a] The source farms are shown in brackets. The farms differ across the states. The farms differ at each sampling occasion.
[b] Ten samples from two farms were not submitted.
[c] Isolates tested positive in both the *mapA* (*C. jejuni*) and *ceuE* (*C. coli*) assays. Isolates amplified in only one allele for MLST SNP HRM typing.
[d] QLD-FR represents Queensland free range.

Table 2.2 Summary of samples collected for the second sampling occasion

Sampling Occasion 2	Samples		Isolates			
	positive	negative	*C. jejuni*[a]	*C. coli*[a]	Unresolved	# samples/farms
QLD	18	2	18 (1,2,3,4)	0	0	20 samples/4 farms
TAS	5	15	5 (2)	0	0	20 samples/4 farms
WA	14	6	13 (1,2,3,4)	1 (2)	0	20 samples/3 farms
SA	20	0	13 (1,2,4)	7 (1,3)	0	20 samples/4 farms
NSW	10	0	5 (2)	2 (1)	3[b] (1)	10 samples/2 farms[c]
QLD-FR[d]	19	1	10 (2,3,4)	9 (1,3)	0	20 samples/4 farms
TOTAL	86	24	64	19	3	

[a] The source farms are shown in brackets. The farms differ across the states. The farms differ at each sampling occasion.
[b] Isolates tested positive in both the *mapA* (*C. jejuni*) and *ceuE* (*C. coli*) assays. Isolates amplified in only one allele for MLST SNP HRM typing.
[c] Ten samples from two farms were not submitted.
[d] QLD-FR represents Queensland free range.

Table 2.3 Summary of samples collected for the third sampling occasion

Sampling Occasion 3	Samples		Isolates			
	positive	negative	*C. jejuni*[a]	*C. coli*[a]	Unresolved	# samples/farms
QLD	20	0	7 (2,4)	13 (1,2,3)	0	20 samples/4 farms
TAS	15	5	15 (2,3,4)	0	0	20 samples/4 farms
WA	20	0	16 (1,2,3,4)	4 (1)	0	20 samples/4 farms
SA	20	0	19 (1,2,3,4)	1 (4)	0	20 samples/4 farms
NSW	20	0	10 (1,2,4)	6 (2,3)	4[b] (4)	20 samples/4 farms
QLD-FR[c]	20	0	11 (2,3,4)	9 (1,4)	0	20 samples/4 farms
TOTAL	115	5	78	33	4	

[a] The source farms are shown in brackets. The farms differ across the states. The farms differ at each sampling occasion.
[b] Isolates tested positive in both the *mapA* (*C. jejuni*) and *ceuE* (*C. coli*) assays. Isolates amplified in only one allele for MLST SNP HRM typing.
[c] QLD-FR represents Queensland free range.

Table 2.4 Summary of samples collected for the fourth sampling occasion

Sampling Occasion 4	Samples		Isolates			
	positive	negative	*C. jejuni*[a]	*C. coli*[a]	Unresolved	# samples/farms
QLD	20	0	5 (4)	15 (1,2,3,4)	0	20 samples/4 farms
TAS	5	15	5 (1)	0	0	20 samples/4 farms
WA	20	0	5 (1,2)	15 (1,2,3,4)	0	20 samples/4 farms
SA	20	0	14 (2,3,4)	6 (1,3)	0	20 samples/4 farms
NSW	20	0	4 (1,3)	6 (2,4)	10[b] (1,2,3)	20 samples/4 farms
QLD-FR[c]	20	0	17 (1,2,3,4)	3 (1)	0	20 samples/4 farms
TOTAL	105	15	50	45	10	

[a] The source farms are shown in brackets. The farms differ across the states. The farms differ at each sampling occasion.
[b] Isolates tested positive in both the *mapA* (*C. jejuni*) and *ceuE* (*C. coli*) assays. Isolates amplified in only one allele for MLST SNP HRM typing.
[c] QLD-FR represents Queensland free range.

Results for the complete year are summarised below in Table 2.5. This shows that of the 465 samples collected, 404 (or 87%) yielded *Campylobacter* spp. For all collection points, except Tas, more than 80% of the samples yielded *Campylobacter* isolates.

Of the 404 samples that tested positive for *Campylobacter* spp., 259 (or 64%) were shown to be *C. jejuni* and 119 (or 29%) were *C. coli*. WA, SA and Qld free range samples have shown a similar result for speciation. Similar results were seen for Vic, with 61% *C. jejuni* and 39% *C. Coli* - but this was only for one sampling occasion (as explained above). Qld samples had a more even distribution, with 51% of samples being *C. jejuni* and 49% *C. coli*. In the Tas samples, 100% of isolates were *C. jejuni*. A unique result was seen in NSW, where less than half of the isolates were shown to be *C. jejuni*. Of the 70 samples that tested positive for *Campylobacter* spp., 30 of these (or 43%) were shown to be *C. jejuni*, 14 (or 20%) were *C. coli* and 26 (or 37%) were classified as unresolved (i.e. they tested positive in both the *mapA* assay for *C. jejuni* and the *ceuE* assay for *C. coli*).

Table 2.5 Summary of samples collected and isolates cultured from one nation-wide company during year one

Year 1	Samples		Isolates			Percentages		
	positive	negative	*C. jejuni*	*C. coli*	Unresolved [b]	*C. jejuni*	*C. coli*	Unresolved
QLD	78	2	40	38	0	51	49	0
TAS	35	45	35	0	0	100	0	0
WA	69	11	49	20	0	71	29	0
SA	70	0	51	19	0	73	27	0
NSW	70	0	30	14	26	43	20	37
VIC	18	2	11	7	0	61	39	0
QLD-FR[b]	64	1	43	21	0	67	33	0
TOTAL	404 (87%)	61	259	119	26	64	29	7

[a] Isolates tested positive in both the *mapA* (*C. jejuni*) and *ceuE* (*C. coli*) assays. Isolates amplified in only one allele for MLST SNP HRM typing.
[b] QLD-FR represents Queensland free range.

Molecular Typing

Detailed results for the MLST SNP HRM typing of the 259 *C. jejuni* and 26 unresolved isolates collected above are presented in Table 2.6. This shows there were 27 MLST SNP HRM types identified in the 259 *C. jejuni* isolates collected nationwide in the first year of the project. Of these 27 MLST SNP HRM types, four types (206, 277, 284 and 310) contained more than 20 isolates (or 8%). Of these four main MLST SNP HRM types, each was found in three or more states. All four of the main MLST SNP HRM types were also seen in free range flocks in Qld.

Table 2.6 Summary of MLST SNP HRM typing results for 259 C. jejuni and 26 unresolved isolates collected from one nation-wide company during year one[a]

MLST SNP HRM Type	No. of Isolates (No. of farms)							No. of isolates	No. of farms
	NSW	SA	WA	TAS	VIC	QLD	QLD-FR[b]		
17	8 (2)	5 (4)	2 (1)			1 (1)		16	8
34		2 (1)		15 (3)				17	4
70				3 (1)				3	1
72		4 (2)						4	2
73			4 (2)					4	2
83						3 (1)		3	1
87	3 (2)							3	2
95		3 (1)			2 (1)	3 (1)		8	3
96		1 (1)	9 (3)	5 (1)			1 (1)	16	6
189		5 (2)	3 (1)	5 (1)		1 (1)	3 (2)	17	7
205		1 (1)						1	1
206		2 (2)	2 (1)		1 (1)	5 (2)	15 (3)	25	9
208						4(1)		4	1
237			1 (1)					1	1
270				5 (1)				5	1
272			1(1)					1	1
277	7 (3)	5 (3)	6 (2)				5 (1)	23	9
284		4 (2)	8 (2)		5 (2)	14 (4)	5 (1)	36	11
289		5 (2)			3 (1)			8	3
301	4 (1)							4	1
310	8 (3)	5 (3)	1(1)			2 (1)	9 (2)	25	10
315		9 (3)				6(2)		15	5
333			11 (5)					11	5
334						1 (1)		1	1
335							5 (1)	5	1
337			1 (1)					1	1
338				2 (1)				2	1
NT-1[c]	26 (7)							26	7

[a] Shading indicates dominant types where there are more than 20 isolates.
[b] Queensland free range samples
[c] NT-1 represents the unresolved or non-typable isolates. Isolates tested positive in both the *mapA* (*C. jejuni*) and *ceuE* (*C. coli*) assays. Isolates amplified in only one allele for MLST SNP HRM typing.

Discussion

In the first year of the project, 87% of the samples collected were positive for *Campylobacter* spp. in the nationwide study. The exception was Tas, where - due to different production conditions - chickens are often processed at an earlier age and therefore less likely to test positive.

Our overall speciation results for the national survey were 64% *C. Jejuni*; 29% *C. Coli*; and 7% classified as unresolved. There was some variation in the species break up between the states.

WA, SA and Qld free range flocks showed the same basic trends as the overall results for *C. jejuni* and *C. Coli*, but without the presence of unresolved isolates. Qld conventional flocks had a more even distribution, with 51% *C. Jejuni*; 49% *C. Coli;* and no unresolved isolates present. The VIC flocks had 61% *C. jejuni* and 39% *C. coli*. Tas differed from all the other states, having 100% *C. jejuni* and no *C. coli* or unresolved isolates. Flocks tested in NSW were 43% *C. jejuni* and 20% *C. Coli,* but had the unique presence of the unresolved isolates representing 37% of the isolates.

There was a diverse range of MLST SNP HRM types present. The biggest type (284) had just 36 isolates representing only 14% of the total *C. jejuni* isolates. There were 10 types (17, 34, 96, 189, 206, 277, 284, 310, 315 and 333) that contained 10 or more isolates. In all cases, with three exceptions (34, 315, 333), the types were obtained from at least four different states. Overall, these results show a wide distribution of genotypes around Australia and no single type dominating.

Whilst there was no single genotype dominating the flocks examined, there was a smaller set of genotypes commonly seen in multiple states. The four dominant types (206, 277, 284, 310) found in the study were all seen in SA, WA and Qld free range flocks. Three of the four dominant types (206, 284 and 310) were seen in Qld conventional flocks and only two dominant types (277 and 310) were seen in NSW. Tas flocks did not test positive to any of the dominant types in the study. These results indicate there may be a common environmental source contaminating flocks in very diverse locations around Australia (SA, WA and Qld free range), such as cattle or wild birds that are carrying these dominant types.

Qld free range flocks consisted of seven different MLST SNP HRM types and four of these were the dominant types (as mentioned above). Only one (335) of the seven types found in free range flocks was unique to free range flocks and consisted of five isolates found only on one farm. The remaining six types that were seen in Qld free range flocks were also observed in at least three other states. The fact that the free range flocks shared common genotypes with conventionally raised flocks suggests that both groups are exposed to similar sources of *Campylobacter*. As the free range flocks have a direct exposure to the external environment (a known reservoir of *Campylobacter*), it would seem that the external environment is also the major source of *Campylobacter* for conventionally raised birds.

Flocks sampled in NSW tested positive to a unique set of isolates that have been classified as unresolved. The unresolved isolates tested positive in both the *C. jejuni* and *C. coli* assays. In addition, they did not amplify in the MLST SNP HRM typing assays for *C. jejuni*. These isolates represented 37% of the total isolates collected in NSW and were almost equivalent to the levels of *C. jejuni* isolated.

Chapter 3: NSW Regional Survey

Introduction

A study was conducted to use MLST SNP HRM typing to compare *C. jejuni* isolates from several companies operating in two intensive production regions in NSW. The first was the Mangrove Mountain region. As described in Chapter 2, this was defined as including farms north of the Hawkesbury River and south of Newcastle - with the majority of farms concentrated in a 20km radius of the locality of Mangrove Mountain.

Samples were collected from four companies rearing meat chickens in the defined locality of Mangrove Mountain. One company provided samples from free range chickens and samples from chickens raised conventionally.

Materials and Methods

Sample Collection

Sampling was performed as described in Chapter 2. Four companies - A, B, C and D - were sampled. On each sampling occasion, four flocks from each company were sampled. Four free range flocks from Company B were also collected. But, due to changes in management practices within Company B, it was only able to supply conventional samples for round one (four farms) and part of rounds two, three and four (one farm per sampling).

Twenty samples were collected from each company on each sampling occasion. Plus 20 samples were collected from Company B's free range flocks. A total of 100 samples was collected on each sampling occasion, meaning 400 samples were collected from Mangrove Mountain for the 12 month period.

Sample Processing

Samples were processed on the day of arrival at the laboratory, as described earlier in the Methodologies sections 'Caecal Samples' and 'Characterisation and Storage of Isolates'.

Molecular Typing

All *C. jejuni* isolates were subjected to MLST SNP HRM typing, as described earlier in the Methodologies sub-section 'MLST SNP HRM typing'.

Results

Detailed results for the four individual sampling occasions are summarised in Tables 3.1, 3.2, 3.3 and 3.4 below. At each sampling occasion, five samples were collected from four different farms. The farms were labelled one, two, three and four for each sampling occasion - but these numbers do not carry over for each sampling occasion. For example, Company A farm one in sampling occasion one may not be the same farm as Company A farm one in sampling occasions two, three or four.

Table 3.1 Summary of samples collected for the first sampling occasion

Sampling Occasion 1		Samples		Isolates			
		positive	negative	*C. jejuni*[a]	*C. coli*[a]	Unresolved[b]	# samples/farms
Company	A	20	0	8 (2,3,4)	0	12 (1,2,3,4)	20 samples/4 farms
	B	19	0	7 (1,2,3)	5 (4)	7 (1,2)	19[c] samples/4 farms
	B-FR[d]	20	0	3 (1,2)	4 (3,4)	13 (1,2,3,4)	20 samples/4 farms
	C	20	0	12 (1,2,3,4)	4 (1)	4 (2)	20 samples/4 farms
	D	18	2	15 (1,2,3,4)	0	3 (3)	20 samples/4 farms
	TOTAL	97	2	45	13	39	

[a] The source farms are shown in brackets. Within each company the farms differ at each sampling occasion.
[b] Isolates tested positive in both the *mapA* (*C. jejuni*) and *ceuE* (*C. coli*) assays. Isolates amplified in only one allele for MLST SNP HRM typing.
[c] One sample was not submitted.
[d] B-FR represents free range samples provided by Company B.

Table 3.2 Summary of samples collected for the second sampling occasion

Sampling Occasion 2		Samples		Isolates			
		positive	negative	*C. jejuni*[a]	*C. coli*[a]	Unresolved[b]	# samples/farms
Company	A	20	0	5 (1)	0	15 (2,3,4)	20 samples/4 farms
	B	5	0	5 (1)	0	0	5[c] samples/1 farm
	B-FR[d]	20	0	3 (1,4)	6 (1,4)	11 (1,2,3)	20 samples/4 farms
	C	20	0	2 (1)	0	18 (1,2,3,4)	20 samples/4 farms
	D	20	0	17 (1,2,3,4)	3 (2)	0	20 samples/4 farms
	TOTAL	85	0	32	9	44	

[a] The source farms are shown in brackets. Within each company the farms differ at each sampling occasion.
[b] Isolates tested positive in both the *mapA* (*C. jejuni*) and *ceuE* (*C. coli*) assays. Isolates amplified in only one allele for MLST SNP HRM typing.
[c] 15 samples/3 farms were not submitted.
[d] B-FR represents free range samples provided by Company B.

Table 3.3 Summary of samples collected for the third sampling occasion

Sampling Occasion 3		Samples		Isolates			
		positive	negative	*C. jejuni*[a]	*C. coli*[a]	Unresolved[b]	# samples/farms
Company	A	20	0	5 (2,4)	0	15 (1,2,3,4)	20 samples/4 farms
	B	5	0	0	3 (1)	2 (1)	5[c] samples/1 farm
	B-FR[d]	20	0	5 (1,2,3)	7 (2,3,4)	8 (1,2,3)	20 samples/4 farms
	C	20	0	15 (2,3,4)	2 (1)	3 (1)	20 samples/4 farms
	D	18[d]	0	13 (1,2,4)	0	5 (3)	18[e] samples/4 farms
	TOTAL	83	0	38	12	33	

[a] The source farms are shown in brackets. Within each company the farms differ at each sampling occasion.
[b] Isolates tested positive in both the *mapA* (*C. jejuni*) and *ceuE* (*C. coli*) assays. Isolates amplified in only one allele for MLST SNP HRM typing.
[c] 15 samples/3 farms were not submitted.
[d] B-FR represents free range samples provided by Company B.
[e] Two samples were not submitted.

Table 3.4 Summary of samples collected for the fourth sampling occasion of the year two NSW survey

Sampling Occasion 4		Samples		Isolates			
		positive	negative	*C. jejuni*[a]	*C. coli*[a]	Unresolved[b]	# samples/farms
Company	A	20	0	5 (3)	0	15 (1,2,4)	20 samples/4 farms
	B	5	0	3 (1)	1 (1)	1 (1)	5[c] samples/1 farm
	B-FR[d]	19	0	11 (1,2,4)	0	8 (1,3)	19[e] samples/4 farms
	C	20	0	16 (1,2,3,4)	1 (4)	3 (3)	20 samples/4 farms
	D	20	0	13 (1,2,3,4)	4 (4)	3 (1)	20 samples/4 farms
TOTAL		84	0	48	6	30	

[a] The source farms are shown in brackets. Within each company the farms differ at each sampling occasion.
[b] Isolates tested positive in both the *mapA* (*C. jejuni*) and *ceuE* (*C. coli*) assays. Isolates amplified in only one allele for MLST SNP HRM typing.
[c] 15 samples/3 farms were not submitted.
[d] B-FR represents free range samples provided by Company B.
[e] One sample was not submitted.

Table 3.5 Summary of samples collected isolates collected in the NSW regional survey

Year 2		Samples		Isolates			Percentages		
		positive	negative	*C. jejuni*	*C. coli*	Unresolved[a]	*C. jejuni*	*C. coli*	Unresolved
Company	A	80	0	23	0	57	29	0	71
	B	34	0	15	9	10	44	26	29
	B-FR[b]	79	0	22	17	40	28	22	51
	C	80	0	45	7	28	56	9	35
	D	76	2	58	7	11	76	9	14
TOTAL		349 (99%)	2	163	40	146	47	11	42

[a] Isolates tested positive in both the *mapA* (*C. jejuni*) and *ceuE* (*C. coli*) assays. Isolates amplified in only one allele for MLST SNP HRM typing.
[b] B-FR represents free range samples provided by Company B.

Results for the complete year are summarised above in Table 3.5. This shows that of the 351 samples collected, 349 (or 99%) yielded *Campylobacter* spp. Companies A, B and C tested *Campylobacter*-positive in 100% of samples provided. Company D submitted the only two (or 0.6%) samples that were *Campylobacter*-negative.

Of the 349 samples that tested positive for *Campylobacter* spp., 47% were shown to be *C. Jejuni*; 11% *C. Coli*; and 42% were classified as unresolved. There was considerable variation in the species breakdown between the four companies and the free range samples from Company B. The unique set of unresolved isolates that were present in year one of the project were again present in year two. The unresolved isolates represent 42% of the isolates collected in this year of the study and are present in all four companies, as well as the free range samples collected from Company B.

Molecular Typing

Detailed results for the MLST SNP HRM typing of the 163 *C. jejuni* and 146 unresolved isolates collected above are present in Table 3.6 below.

Table 3.6 Summary of genotyping results of 163 C. jejuni and 146 unresolved isolates from the NSW regional surveya

Genotype	No. of Isolates (No. of farms)					No. of isolates	No. of farms
	A	B	B-FR[b]	C	D		
17	6 (2)			10 (3)	6 (2)	22	7
73					6 (4)	6	4
111			1 (1)			1	1
186				12 (3)		12	3
206		1 (1)	1 (1)	1(1)		3	3
237			1 (1)			1	1
272		3 (1)				3	1
277	2 (1)	3 (3)	13 (7)	4 (2)	15 (6)	37	19
284	2(1)					2	1
297			1 (1)		5 (1)	6	2
301	5 (2)	5 (2)	3 (3)	2 (1)	11 (3)	26	11
309	5 (1)	1 (1)				6	2
310	3 (3)	2 (2)	2 (2)	11 (3)	1 (1)	19	11
311					3 (1)	3	1
315				5 (3)	10 (2)	15	5
335					1(1)	1	1
NT-1[c]	57 (14)	10 (4)	40 (11)	28 (7)	11 (3)	146	39

[a] Shading indicates dominant types where there is greater than 16 isolates
[b] Company B provided free range samples
[c] NT-1 represents the unresolved or non-typable isolates. Isolates tested positive in both the *mapA* (*C. jejuni*) and *ceuE* (*C. coli*) assays. Isolates amplified in only one allele for MLST SNP HRM typing.

Results presented in Table 3.6 showed there were 16 MLST SNP HRM types identified in the 163 *C. jejuni* isolates collected in the NSW regional survey in year two of the project. Of these 16 MLST SNP HRM types, four types (17, 277, 301, 310) contained more than 16 isolates (or 10%). Of the four main MLST SNP HRM types, each was found in three or more companies. Three (277, 301, 310) of the four main MLST SNP HRM types were also seen in free range flocks from Company B. The unresolved, or non-typable group one, was by far the most prevalent single type - representing 42% of the isolates and seen in all four companies and in free range flocks.

Discussion

Of the samples collected, 99% were positive for *Campylobacter* spp. in the NSW regional survey for the second year of the project. With such a high rate of positive samples, there was no variation in the positive rate between the companies or the free range flocks.

Our overall speciation results for the NSW regional survey were 47% *C. Jejuni*; 11% *C. Coli;* and 42% were classified as unresolved. The unresolved isolates will be discussed shortly.

There was considerable variation in the species breakdown between the four companies. Company C, with 56% *C. jejuni* and 9% *C. coli*, was the only company to have the same basic trend as the overall results. Company D showed an expected level of 76% *C. jejuni* and 9 % *C. coli*. Company A conventional flocks and Company B free range flocks showed a similar level of *C. Jejuni,* with 29% and 28% respectively. But they differed in that Company A had no *C. coli* present, whereas Company B free range showed 22% *C. coli*. Company B conventional flocks showed unique results - with 44% *C. jejuni* and 26% *C. coli*.

There was a range of MLST SNP HRM types present. The most common type (277) had 37 isolates representing 23% of the total *C. jejuni* isolates. There were four types (17, 277, 301, 310) that contained more than 16 isolates. In all cases - except one (17) - the types were obtained from all four companies, as well as from free range flocks in Company B. Within each company there was a similar distribution of genotypes, ranging from six to eight different genotypes present.

Overall, these results show a wide distribution of genotypes in this regional area of NSW and no single *C. jejuni* type dominating.

Although there was no dominant *C. jejuni* type, all companies in NSW - including the free range flocks - tested positive to the unique set of isolates that have been classified as unresolved. These unresolved isolates were identified in NSW flocks in the national survey in the first year of the project. In this more focused NSW regional survey in year two, the unresolved isolates represented 42% of the total isolates collected. There was a lot of variation in their presence within the individual companies. Company D had the lowest level, with only 14% unresolved. Company B and Company C had similar levels of 29% and 35% respectively. Company B free range had a level of 51% and Company A had the highest level, with 71% unresolved isolates present.

These findings that there are a variety of genotypes and substantial numbers of unresolved isolates present give added weight to the theory that poultry flocks are indeed contaminated from the external environment by horizontal transmission.

It would appear in the Mangrove Mountain region of NSW there is a very common external source, such as wild birds or a wild animal population that are resident in the area, that are contaminating poultry flocks across the four different companies by horizontal transmission. Company B free range flocks consisted of seven different MLST SNP HRM types, with three of these being the dominant types (277, 301, 310). There were two SNP MLST HRM types (111, 237) that were only found in free range flocks and not conventionally raised flocks. But in each case, they were only single isolates during a 12 month period and not considered significant. The majority of types found in free range flocks were genotypes shared with conventionally raised flocks, suggesting that both groups are exposed to similar sources of *Campylobacter* (as discussed in Chapter 2 as part of the national survey).

There were six MLST SNP HRM types (111, 186, 237, 272, 284, 311) where each type was only found in one company. Only one type (186) in Company C was found in reasonable numbers (12 isolates from three farms). The remaining five types (111, 237, 272, 284, 311) were unique to a particular company, ranged from one to three isolates, only found on one farm each and not considered significant.

Chapter 4: Qld Regional Survey

Introduction

A study was conducted to use MLST SNP HRM typing to compare *C. jejuni* isolates from several companies operating within two intensive production regions in this state. The second was the South East Brisbane region. As described in Chapter 2, this included farms rearing chickens in the Redland Bay and Beaudesert areas of Qld.

Samples were collected from three companies that were rearing meat chickens in the South East Brisbane region. One company provided free range samples and samples from chickens raised conventionally.

Materials and Methods

Sample Collection

Sampling was performed as described in Chapter 2. Three companies, A, B, and C were sampled. The company codes are specific for this Chapter. That is, Company A in this chapter is not necessarily Company A in Chapter 3. On each sampling occasion, four flocks from each processing facility in each company were sampled. Samples from four free range flocks from Company C were also collected.

Twenty samples were collected from each company on each sampling occasion and 20 samples were collected from Company C free range flocks. Eighty samples were collected on each sampling occasion, meaning a total of 320 samples were collected from the South East Brisbane Region for the 12 month period.

Sample Processing

Samples were processed on the day of arrival at the laboratory, as described earlier in the Methodologies sections ‘Caecal Samples’ and ‘Characterisation and Storage of Isolates’.

Molecular and Typing

All *C. jejuni* isolates were subjected to MLST SNP HRM typing, as described earlier in the Methodologies sub-section ‘MLST SNP HRM typing’.

Results

Detailed results for the four individual sampling occasions are summarised in Tables 4.1, 4.2, 4.3 and 4.4 below. At each sampling occasion, five samples were collected from four different farms. The farms were labelled one, two, three and four for each sampling occasion - but these numbers did not necessarily carry over for each sampling occasion. For example, Qld farm one in sampling occasion one may not be the same farm as Qld farm one in sampling occasions two, three or four.

Table 4.1 Summary of samples collected for the first sampling occasion

Sampling Occasion 1		Samples		Isolates			
		positive	negative	*C. jejuni*[a]	*C. coli*[a]	Unresolved[b]	# samples/farms
Company	A	20	0	11 (1,2,3,4)	9 (1,2,3)	0	20 samples/4 farms
	B	19	0	18 (1,2,3,4)	1 (1)	0	19[c] samples/4 farms
	C	16	4[d]	6 (3,4)	10 (1,2)	0	20 samples/4 farms
	C-FR[e]	20	0	7 (1,2,3,4)	10 (2,3,4)	3 (1)	20 samples/4 farms
	TOTAL	75	4	42	30	3	

[a] The source farms are shown in brackets. Within each company the farms differ at each sampling occasion.
[b] Isolates tested positive in both the *mapA* (*C. jejuni*) and *ceuE* (*C. coli*) assays. Isolates amplified in only one allele for MLST SNP HRM typing.
[c] One sample was not submitted.
[d] Four *Helicobacter* isolates were found on Farm 4.
[e] Free range samples provided by Company C.

Table 4.2 Summary of samples collected for the second sampling occasion

Sampling Occasion 2		Samples		Isolates			
		positive	negative	*C. jejuni*[a]	*C. coli*[a]	Unresolved[b]	# samples/farms
Company	A	20	0	11 (1,2,4)	9 (2,3,4)	0	20 samples/4 farms
	B	15	5	10 (2,4)	5 (1)	0	20 samples/4 farm
	C	20	0	8 (1,4)	10 (1,2,3)	2 (2)	20 samples/4 farms
	C-FR[c]	20	0	9 (1,3,4)	11 (1,2,3)	0	20 samples/4 farms
	TOTAL	75	5	38	35	2	

[a] The source farms are shown in brackets. Within each company the farms differ at each sampling occasion.
[b] Isolates tested positive in both the *mapA* (*C. jejuni*) and *ceuE* (*C. coli*) assays. Isolates amplified in only one allele for MLST SNP HRM typing.
[c] Free range samples provided by Company C.

Table 4.3 Summary of samples collected for the third sampling occasion

Sampling Occasion 3		Samples		Isolates			
		positive	negative	*C. jejuni*[a]	*C. coli*[a]	Unresolved[b]	# samples/farms
Company	A	20	0	12 (1,2,3,4)	8 (1,2,3)	0	20 samples/4 farms
	B	20	0	3 (1)	17 (1,2,3,4)	0	20 samples/4 farm
	C	20	0	2 (3)	13 (1,2,3)	5 (4)	20 samples/4 farms
	C-FR[c]	20	0	11 (1,2,4)	4 (2)	5 (3)	20 samples/4 farms
	TOTAL	80	0	28	42	10	

[a] The source farms are shown in brackets. Within each company the farms differ at each sampling occasion.
[b] NT-1 represents the unresolved or non-typable isolates. Isolates tested positive in both the *mapA* (*C. jejuni*) and *ceuE* (*C. coli*) assays. Isolates amplified in only one allele for MLST SNP HRM typing.
[c] Free range samples provided by Company C.

Table 4.4 Summary of samples collected for the fourth sampling occasion

Sampling Occasion 4		Samples		Isolates			
		positive	negative	*C. jejuni*[a]	*C. coli*[a]	Unresolved[b]	# samples/farms
Company	A	20	0	7 (1,2,3,4)	13 (1,2,3,4)	0	20 samples/4 farms
	B	19	0	9 (3,4)	10 (1,2)	0	19[c] samples/4 farm
	C	20	0	10 (2,3,4)	7 (1,4)	3 (2)	20 samples/4 farms
	C-FR[c]	20	0	9 (3,4)	1 (4)	10 (1,2)	20 samples/4 farms
	TOTAL	79	0	35	31	13	

[a] The source farms are shown in brackets. Within each company the farms differ at each sampling occasion.
[b] Isolates tested positive in both the *mapA* (*C. jejuni*) and *ceuE* (*C. coli*) assays. Isolates amplified in only one allele for MLST SNP HRM typing.
[c] One sample was not submitted.
[d] Free range samples provided by Company C.

Table 4.5 Summary of samples collected isolates collected in the QLD regional survey

Year 3		Samples		Isolates			Percentages		
		positive	negative	*C. jejuni*	*C. coli*	Unresolved[a]	*C. jejuni*	*C. coli*	Unresolved
Company	A	80	0	41	39	0	51	49	0
	B	73	5	40	33	0	55	45	0
	C	76	4	26	40	10	34	53	13
	C-FR[b]	80	0	36	26	18	45	33	23
	TOTAL	309 (97%)	9	143	138	28	46	45	9

[a] Isolates tested positive in both the *mapA* (*C. jejuni*) and *ceuE* (*C. coli*) assays. Isolates amplified in only one allele for MLST SNP HRM typing.
[b] Free range samples provided by Company C.

Results for the complete year are summarised above in Table 4.5. This shows that of the 318 samples collected, 309 (or 97%) yielded *Campylobacter* spp. Company A and Company C free-range samples were 100% *Campylobacter*-positive. Company B submitted samples that were 94% positive and Company C's samples were 95% positive for *Campylobacter* spp.

Of the 309 samples that tested positive for *Campylobacter* spp., overall 46% were shown to be *C. Jejuni*; 45% *C. Coli;* and 9% were classified as unresolved. Companies A and B showed similar results for speciation, with close to a 50:50 *C. jejuni/coli* ratio. Company C had a lower level of *C. Jejuni*, with only 34%, and about the same level of *C. Coli*, with 53%. Company C free range had 45% *C. jejuni* and a lower level of *C. coli* at 33%. The unique collection of isolates classified as unknown in NSW was also seen this year in Qld. Conventional flocks had 10 (or 13%) and free range flocks had 18 (or 23%) unresolved isolates.

Molecular Typing

Detailed results for the MLST SNP HRM typing of the 143 *C. jejuni* isolates collected above are presented in Table 4.6 below.

Table 4.6 Summary of genotyping results of 143 C. jejuni and 28 unresolved isolates from the QLD regional surveya

Genotype	No. of Isolates (No. of farms)				No. of isolates	No. of farms
	A	B	C	C –FR[b]		
17		4 (1)	2 (1)	2 (1)	8	3
96				1 (1)	1	1
189			3 (1)	1 (1)	4	2
206	2 (2)	6 (3)	8 (3)	2 (1)	18	9
272				1 (1)	1	1
277	32 (10)	4 (2)		9 (2)	45	14
284			6 (2)	5 (2)	11	4
297	1 (1)				1	1
309	2 (2)				2	2
310	4 (2)	19 (5)	6 (2)	7 (4)	36	13
311				5 (1)	5	1
315		3 (2)		3 (1)	6	3
334		4 (1)			4	1
336			1 (1)		1	1
NT[c]-1			10 (3)	18 (4)	28	7

[a] Shading indicates dominant types where there is greater than 14 isolates
[b] Company C free range samples
[c] NT-1 represents the unresolved or non-typable isolates. Isolates tested positive in both the *mapA* (*C. jejuni*) and *ceuE* (*C. coli*) assays. Isolates amplified in only one allele for MLST SNP HRM typing.

Table 4.6 shows there were 14 MLST SNP HRM types identified in the 143 *C. jejuni* isolates collected in the Qld regional survey in year three of the project. Of these 14 MLST SNP HRM types, three types (206, 277, 310) contained more than 14 isolates (or 10%). Of the three main MLST SNP HRM types, each was found in at least two of the companies in Qld. All three of the main MLST SNP HRM types were also seen in the free range flocks from Company C. The unresolved, or non-typable group one, was also found in Qld - with 28 isolates found in Company C only (in conventionally raised and free range flocks).

Discussion

Of samples collected, 97% were positive for *Campylobacter* spp. in the Qld regional survey in the second year of the project. There was slight variation between the companies in Qld. Company A and Company C free range flocks tested 100% positive and flocks from Companies B and C had comparable levels of 93% and 94% respectively.

Our overall speciation results for the Qld regional survey were 46% *C. Jejuni*; 45% *C. Coli;* and 9% were classified as unresolved. There was only slight variation in the species breakdown between the three companies. Companies A and B had similar results of 51 and 55% *C. Jejuni;* and 49% and 45% *C. coli* respectively. Compared with Companies A and B, Company C's conventionally raised birds showed a lower level of *C. jejuni* (34%). This was about the same level of *C. coli* plus the presence of the unresolved isolates. These unresolved isolates were seen in NSW in years one and two of the project. Compared with Companies A and B, Company C's free range flocks had a lower level of *C. coli* (33%) but a higher level of the unresolved isolates (23%).

There was a range of MLST SNP HRM types present, with the largest type (277) having 45 isolates representing 31% of the total *C. jejuni* isolates. There were three types (206, 277, 310) that contained more than 14 isolates. In two (206, 310) out of these three types, isolates were obtained from all three companies as well as from free range flocks from Company C. Within each company, there were five different genotypes present during the year in conventionally raised chickens.

In contrast to the NSW results, Company A had one type (277) dominant that represented 78% of the isolates. Within Company B, one type (310) was dominant and represented 53% of the isolates. Company C had a more even distribution of genotypes in the conventionally raised flocks and the free range flocks. The free range flocks had twice as many genotypes, with10 different genotypes plus the unresolved isolates.

Overall, these results show a wide distribution of genotypes in this regional area of Qld. Compared to NSW, in two out of the three Qld companies one *C. jejuni* type has dominated within each company.

As noted above, Company C free range flocks consisted of 10 different MLST SNP HRM types and three of these are the dominant types (206, 277, 310). There were three SNP MLST HRM types (96, 272, 311) that were only found in the free range flocks and not in conventionally raised flocks. Of these three types, two (96, 272) were only single isolates during a 12 month period and the third type (311) consisted of only five isolates from one farm. Therefore, these three types are not considered significant.

Overall, the majority of types found in free range flocks were genotypes shared with conventionally raised flocks. This suggests that both groups are exposed to similar sources of *Campylobacter* (as discussed in Chapter 2 as part of the national survey).

Chapter 5: Variation in genotypes over time

Introduction

The project was designed to study the variation in genotypes in two regional areas across two time periods. Isolates from NSW were compared in years one and two and isolates from Qld were compared in years one and three of the project.

As outlined in Chapter 2, the collection of samples from NSW for the national survey focused on chickens reared in the Mangrove Mountain region. For this study, Mangrove Mountain has been defined as including farms north of the Hawkesbury River and south of Newcastle - with the majority of farms concentrated in a 20km radius of the locality of Mangrove Mountain.

The collection of samples from Qld for the national survey was focused in the Beaudesert and Redland Bay areas, defined as the South-East Brisbane region.

Material and Methods

Samples

NSW Regional Survey

The genotypes of 30 *C. jejuni* isolates collected and typed in year one of the project (presented in Chapter 2, Table 2.6) will be compared with the 45 *C. jejuni* isolates collected and typed in year two of the project (presented in Chapter 3, Table 3.6)

QLD Regional Survey

The genotypes of 83 *C. jejuni* isolates (40 conventional and 43 free range) collected and typed in year one of the project (presented in Chapter 2, Table 2.6) will be compared with the 62 *C. jejuni* isolates (26 conventional and 36 free range) collected and typed in year three of the project (presented in Chapter 3, Table 4.6)

Results

NSW Regional Survey

Detailed results comparing the MLST SNP HRM types found in *C. jejuni* isolates from years one and two of the project are presented in Table 5.1 below. This shows that four types (17, 277, 301, 310) were found in both years of the study. The isolates classified as unresolved, or NT-1, were also found in both years of the study - 26 in year one from seven flocks and 28 in year two from seven flocks.

QLD Regional Survey

Detailed results comparing the MLST SNP HRM types found in *C. jejuni* isolates from years one and three of the project are presented in Table 5.2 below. This shows that for conventionally raised flocks, there were five types (17, 189, 206, 284, 310) and for free range flocks, there were six types (96, 189, 206, 277, 284, 310) found in both years of the study.

Table 5.1 Comparison of genotypes collected in NSW from years one and two of the study

Genotypes	Year	No. of Isolates (No. of farms)				
		A	B	B-FR[a]	C	D
17[b]	1				**8 (2)**	
	2	6 (2)			**10 (3)**	6 (2)
73	2					6 (4)
87	1				3 (2)	
111	2			1 (1)		
186	2				12 (3)	
206[c]	2		1 (1)	1 (1)	1 (1)	
237	2			1 (1)		
272	2		3 (1)			
277[b c]	1				**7 (3)**	
	2	2 (1)	3 (3)	13 (7)	**4 (2)**	15 (6)
284[c]	2	2 (1)				
297	2			1 (1)		5 (1)
301[b]	1				**4 (1)**	
	2	5 (2)	5 (2)	3 (3)	**2 (1)**	11 (3)
309	2	5 (1)	1 (1)			
310[b c]	1				**8 (3)**	
	2	3 (3)	2 (2)	2 (2)	**11 (3)**	1 (1)
311	2					3 (1)
315	2				5 (3)	10 (2)
NT[d]-1	1				**26 (7)**	
	2	57 (14)	10 (4)	40 (11)	**28 (7)**	11 (3)

[a] Company B provided free range samples.
[b] Dominant types, meaning more than 16 isolates, found in the NSW survey in year two of the project.
[c] Dominant types, meaning more than 20 isolates, found in the national survey in year one of the project.
[d] NT-1 represents the unresolved or non-typable isolates. Isolates tested positive in both the *mapA* (*C. jejuni*) and *ceuE* (*C. coli*) assays. Isolates amplified in only one allele for MLST SNP HRM typing.

Table 5.2 Comparison of genotypes collected in QLD from years one and three of the study

Genotype	Year	No. of Isolates (No. of farms)			
		A	B	C	C -FR[a]
17	1			**1 (1)**	
	3		4 (1)	**2 (1)**	2 (1)
83	1			3 (1)	
95	1			3 (1)	
96	1				**1 (1)**
	3				**1 (1)**
189	1			**1 (1)**	**3 (2)**
	3			**3 (1)**	**1 (1)**
206[b c]	1			**5 (2)**	**15 (3)**
	3	2 (2)	6 (3)	**8 (3)**	**2 (1)**
208	1			4 (1)	
272	3				1 (1)
277[b c]	1				**5 (1)**
	3	30 (10)	4 (2)		**9 (2)**
284[c]	1			**14 (4)**	**5 (1)**
	3			**6 (2)**	**5 (2)**
297	3	1 (1)			
309	3	2 (2)			
310[b c]	1			**2 (1)**	**9 (2)**
	3	4 (2)	19 (5)	**6 (2)**	**7 (4)**
311	3				5 (1)
315	3		3 (2)		3 (1)
334	3		4 (1)		
336	3			1 (1)	
NT[d]-1	3			10 (3)	18 (4)

[a] Company C provided free range samples.
[b] Dominant types, meaning more than 16 isolates, found in the NSW survey in year two of the project.
[c] Dominant types, meaning more than 20 isolates, found in the national survey in year one of the project.
[d] NT-1 represents the unresolved or non-typable isolates. Isolates tested positive in both the *mapA* (*C. jejuni*) and *ceuE* (*C. coli*) assays. Isolates amplified in only one allele for MLST SNP HRM typing.

Discussion

Overall, there were 16 MLST SNP HRM types found in NSW during years one and two of the study and unresolved isolates. Of these 16 types, four (17, 277, 301, 310) were found in both years of the study along with the unresolved isolates. Two (277, 310) of these four types were dominant in year one and all four (17, 277, 301, 310) were dominant in year two of the study. The unresolved isolates were seen in both years of the study in NSW.

In Qld conventional flocks, there were nine MLST SNP HRM types found during years one and three of the study. Of these nine types, five (17, 189, 206, 284, 310) types were found in both years of the study. Three (206, 284, 310) of these five types were dominant in year one of the study and two (206, 310) were dominant in year two.

In the Qld free range flocks, there were 10 MLST SNP HRM types found during years one and three of the study. Of these 10 types, six (96, 189, 206, 277, 284, 310) types were found in both years. Four (206, 277, 284, 310) of these six types were dominant in all flock types in year one and three (206, 277, 310) were dominant in all flock types in year two of the study.

Of particular interest in the Qld study is the emergence of the unresolved, or NT-1, isolates that were previously only seen in NSW in the first two years of the study. Not only did these isolates emerge in Qld, but they became the dominant type in both conventional and free range flocks.

Chapter 6: Human investigations

Introduction

During this project, two sets of isolates became available for investigation. One set consisted of *Campylobacter* isolates from human campylobacteriosis cases in Sydney from August 2010 to March 2011. The other set came from an investigation of a big outbreak of human campylobacteriosis in the NQ population by the Queensland Health Department.

Material and Methods

Sample Collection

NSW Human Isolates

Faecal samples were collected from patients presenting with gastroenteritis to the emergency departments of four Sydney hospitals - Westmead, Mount Druitt, Blacktown and Auburn. *Campylobacter* spp. were isolated and stored at -20ºC at Westmead Hospital. The isolates were from human cases of campylobacteriosis between August 2010 and March 2011.

Qld Poultry Isolates Associated with NQ Human Campylobacter Outbreak

Fifty two viable *Campylobacter* spp. cultures were received on SBA plates from the Qld Health Department. Samples were collected from retail poultry products of a single company in NQ from January to March 2012.

Sample Processing

NSW Human Isolates

Initial work at the Westmead Hospital showed that the stored frozen cultures of *Campylobacter* spp. were not viable. The collection consisted of 1.5 mL ampoules of storage media. The collection was shipped to the Ecosciences Precinct. Each sample was transferred to a 1.7 mL centrifuge tube and centrifuged at 16 000 x g for 5 mins. The supernatant was removed and purified DNA was prepared from the pellet using PrepMan Ultra Sample Preparation Reagent (Applied Biosystems). The extracted DNA was stored at -20ºC.

QLD Poultry Isolates Associated with NQ Human Campylobacter Outbreak

Each isolate was subjected to two single colony subcultures for purification prior to storage. Isolates were then characterised and stored as described earlier in the Methodologies section ‘Characterisation and Storage of Isolates’.

Molecular Typing

All *C. jejuni* isolates were subjected to MLST SNP HRM typing as described earlier in the Methodologies sub-section ‘MLST SNP HRM typing’.

Results

NSW Human Isolates

Of the 50 *Campylobacter* spp. isolates tested, 42 (or 84%) were *C. jejuni* and 8 (or 16%) were *C. coli*.

Detailed results for the MLST SNP HRM typing of the 42 *C. jejuni* human isolates collected in NSW are presented in Table 6.1 below. This shows that there were 18 MLST SNP HRM types identified from humans in NSW. Eight of these 18 types (shaded below in Table 6.1) have been seen in poultry flocks from NSW in years one and two of our project. Two (206, 310) of these eight types were dominant in year one and three (17, 277, 310) were dominant in year two of the study.

Table 6.1 Summary of MLST SNP HRM typing results for 42 C. jejuni human isolates from NSW between August 2010 and March 2011a

MLST SNP HRM Type	No. of Isolates	Percentage
17[b]	5	12
34	2	5
49	1	2
70	1	2
72	2	5
87	1	2
96	2	5
100	3	7
112	1	2
189	4	10
206[c]	4	10
221	1	2
277[b]	2	5
294	1	2
309	2	5
310 [b c]	9	21
311	1	2
315	1	2

[a] Shading indicates types that have been shown in poultry in this study over the same time period.
[b] Dominant types found in the NSW survey in year two of the project.
[c] Dominant types found in the national survey in year one of the project.

Qld Poultry Isolates Associated with NQ Human Campylobacter Outbreak

Of the 52 *Campylobacter* spp. isolates tested, 45 (or 87%) were *C. jejuni* and 7 (or 13%) were *C. coli*.

Detailed results for the MLST SNP HRM typing of the 45 *C. jejuni* isolates collected in NQ are presented in Table 6.2 below. This shows there were six MLST SNP HRM types identified from the NQ poultry isolates. One (206 - shaded row in Table 6.2) of the six types identified has been seen in poultry flocks from Qld in years one and three of the study. The one type (206) that is common between the NQ poultry isolates and the other poultry isolates around Australia was also a dominant genotype in both year one (national survey) and year three (Qld survey) of the study.

Table 6.2 Summary of MLST SNP HRM typing results for 45 C. jejuni isolates from NQ between January and March 2012.

MLST SNP HRM Type	No. of Isolates	Percentage
34	8	18
96	2	4
206[a b c]	31	69
270	1	2
272	1	2
297	2	4

[a] Shading indicates types that have been shown in poultry in this study over the same time period.
[b] One of the dominant types found in the national survey in year one of the study.
[c] One of the dominant types found in QLD in year three of the study.

Discussion

There was a considerable amount of variation seen in the NSW human isolates, with 18 MLST SNP HRM types observed in the 42 *C. jejuni* samples received. Of these 18 types, eight (17, 87, 206, 277, 309, 310, 311, 315) types were observed in poultry in NSW during years one and two of the project. Of the eight types observed in the poultry and human isolates from NSW, two (206, 310) were dominant types in poultry in year one and three (17, 277, 310) were dominant in year two. Interestingly, these eight types that are common to poultry represent 59% of the human *C. jejuni* isolates tested. More than 30% of the human isolates were types 17 and 310, which were two of the dominant types in the extensive NSW survey in year two of the project.

The unresolved, or NT-1, isolates were not observed in this collection of human isolates from NSW. Even if we had observed a sample in this collection testing positive in both the *mapA* (*C. jejuni*) and the *ceuE* (*C. coli*) assays, caution would need to be taken when interpreting the results. These samples were DNA samples from non-viable cultures. As we played no role in the isolation and storage of these isolates, we would have to exhibit caution as to whether the cultures had been subjected to the rigorous purification process we used on all other isolates (two sequential single colony picks). A double positive result might mean DNA has been prepared from a sample that was a mixed culture of *C. jejuni* and *C. coli*.

Of the 45 *C. jejuni* isolates collected from poultry in NQ, there were six SNP MLST HRM types observed. Of these six types, only one (206) was observed in poultry in years one and three of the project. This type (206) was one of the dominant types in poultry in year one, where it was observed in conventional and free range flocks. It was also dominant in year three of the project and was observed in all three companies in SEQ - in both conventional and free range flocks from Company C.

Importantly, 69% of the 45 *C. jejuni* isolates tested from NQ poultry were shown to be MLST SNP HRM type 206. The dominance of one type in these NQ poultry isolates is in contrast to almost all our other results during the project. We did show a domination of type 277 (78%) in Company A in year three. But the NQ isolates are from retail poultry products, whereas all the other samples had been from the caeca of individual birds at the start of processing. This is an unusual finding, as a previous study by Colles *et al*., (2008) reported that processing normally results in an increased genotypic diversity.

Chapter 7: Overall Discussion

Our strategy to collect samples from older birds, above 40 days of age, to maximise *Campylobacter*-positive samples was successful. Of the samples collected, 87% were positive for *Campylobacter* spp. in the national study in the first year of the project. The exception was Tas, where different production conditions mean chickens are often processed at an earlier age and therefore are less likely to test positive - as noted extensively in the literature (Jacobs-Reitsma *et al*. 1995; Evans and Sayers 2000; Lawes *et al*. 2012). Only 44% of samples from Tasmania yielded *Campylobacter* spp. In the NSW regional survey, in year two of the project, 99% of samples tested positive and in the Qld regional survey in year three of the project, 97% of samples tested positive for *Campylobacter* spp.

Our overall speciation results for the national survey were 64% *C. Jejuni;* 29% *C. Coli;* and 7% were classified as unresolved. In comparison, a big UK study conducted over a three-year period that examined 930 *Campylobacter*-positive batches of broilers reported 75% *C. jejuni* and 25% *C. coli* (Lawes *et al*. 2012). One limitation of the UK study was only one isolate per positive batch of broilers was examined. This means the UK study could not recognise the presence of multiple species. It has been shown in another study (Rodgers and Vidal, 2009) that 21.6% of UK batches are colonised with both *C. jejuni* and *C. coli*.

Our national survey in year one in Australia shows a comparable rate of dual colonisation, with 19.3% of positive farms testing positive to both *C. jejuni* and *C. coli*. Our data is also based on examination of five isolates from each batch of broilers across 83 *Campylobacter*-positive batches of broilers.

The rate of dual colonisation was quite variable across the three years of the study. In the NSW regional study (year two), 12.6% of positive flocks tested positive to both *C. jejuni* and *C. coli*. In the Qld regional study (year three), a rate of 37.5% was observed. This year three level is above the rate (25%) for Qld flocks in the national survey (year one).

As noted in Chapter 2, there was some variation observed in the species composition between the states in the national survey (see Figure 7.1 below). Some of the states showed the same basic trends as the overall results. Other states, such as NSW and Qld, showed a much lower level of *C. jejuni* (as shown in Figure 7.1 below). In particular, NSW had only 43% *C. Jejuni,* which was very similar to the overall level of *C. jejuni* seen in the second year of the study during the NSW regional survey. In addition, Qld conventional flocks had almost equal levels of *C. jejuni* and *C. coli* and this trend was also reflected in the Qld regional survey in the third year of the project. Tas had 100% *C. jejuni*.

Figure 7.1 Speciation results for the overall national survey and for each state

In the NSW regional survey, there was a slight increase in the overall level of *C. jejuni* (47%) observed compared with levels in NSW in the national survey (43%). A decrease in the overall level of *C. coli* was also observed (20% down to 11%). Also, we observed a slight increase in the overall level of the unresolved isolates from 37% in year one to 42% in year two. While the difference in the overall increase of unresolved isolates was slight, at the company level there was considerable variation in the levels – ranging from 14% to 71% (as shown in Figure 7.2 below).

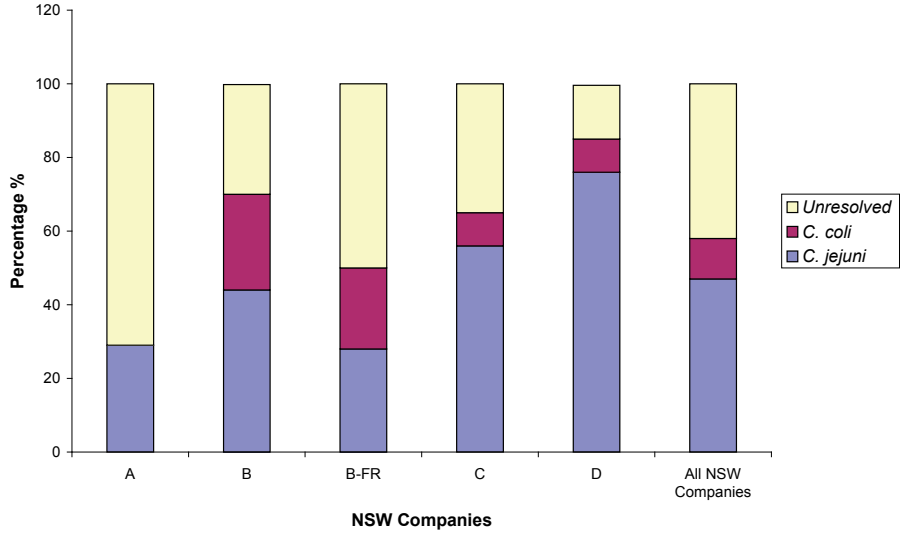

Figure 7.2 Speciation results for the overall NSW survey (year two) and for each company

The Qld regional survey in year three of the project showed a reduction in the overall level of *C. jejuni* (46%) compared with levels in Qld in the national survey (51% conventional and 67% free range flocks). A marked increase was observed for *C. coli* (29% to 45%) and a slight increase for the unresolved isolates (7% to 9%). But there is a more noticeable variation when results are broken down to company level in the Qld regional survey (as shown in Figure 7.3 below). For instance, Companies A and B had similar levels of *C. jejuni* and *C. coli* as the overall year three results and with no unresolved isolates present. In comparison, Company C conventional flocks had a lower level of *C. jejuni* (34%) than year one (51%); a slightly higher level of *C. coli* (53%) than year one (49%); and the presence of the unresolved isolates (13%). Likewise, Company C free range flocks also differed, with a lower level of *C. jejuni* (45%) than year one (67%); the same level of *C. coli* (33%) as year one; and the presence of unresolved isolates (23%).

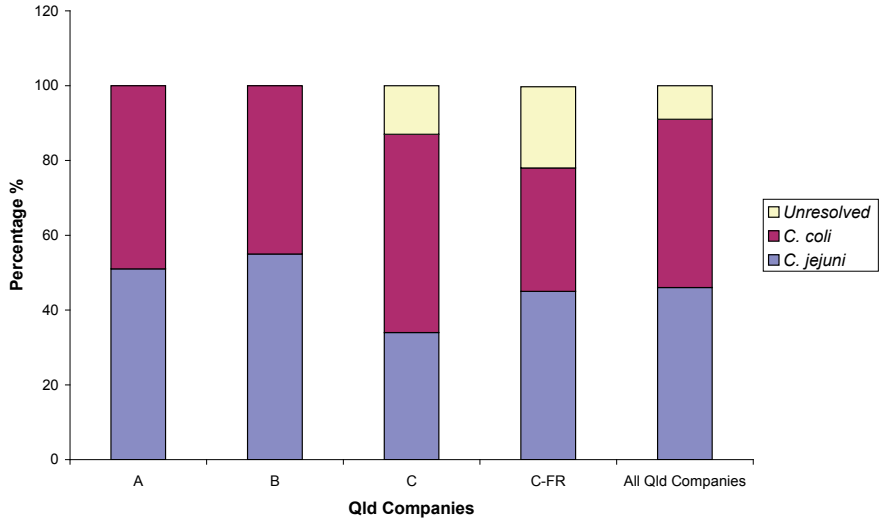

Figure 7.3 Speciation results for the overall Qld survey (year three) and for each company

There was a diverse range of MLST SNP HRM types present across Australia,(as shown in Figure 7.4) and in the regional surveys in NSW and Qld (shown in Figures 7.5 and 7.6 respectively). The number of genotypes observed was varied - across Australia there were 27 and between the States there were 16 in NSW and 14 in Qld. But in each survey there were still only a few dominant types found – four nationally, four in NSW and three in Qld. - and no one type was more than 31% of the isolates for that particular survey.

Overall, these results show a wide distribution of genotypes across Australia and within a small geographical area where multiple companies are present - with no one type dominating. This finding suggests that vertical transmission is not associated with *Campylobacter* colonisation of broiler flocks. Many overseas studies have also reported similar findings (Newell and Fearnley 2003).

While no one genotype dominated, in the national survey or both regional surveys, there was a smaller set of types commonly seen in multiple States or across multiple companies. For example, in the national survey the four dominant types (206, 277, 284, 310) found in the study were all seen in SA, WA and Qld free range flocks. Three of the four dominant types (206, 284, 310) were seen in Qld conventional flocks and only two dominant types (277, 310) were seen in NSW. Tasmanian flocks did not test positive to any of the dominant types in the study.

These results indicate there may be a common environmental source contaminating flocks in very diverse locations around Australia (SA, WA and Qld free range), such as cattle or wild birds that are carrying these dominant types. It is commonly known that other animals harbour *C. jejuni* as natural intestinal flora, making them potential contaminants for poultry flocks (Schouls *et al*. 2003).

Similarly, there are dominant types in the regional surveys that occur across companies. For example, in NSW, three of the four dominant types (277, 301, 310) were obtained from all four companies and from free range flocks. These results again suggest there may be a common environmental source contaminating flocks from different companies. This trend was repeated in the Qld survey.

A unique finding in the Qld regional survey was the marked dominance of one genotype in one company. In Company A, 78% of isolates were a single genotype (Type 277). In the other companies (B, C and C free range), no single genotype reached 50% dominance. Importantly, type 277 was detected at all four samplings and on multiple farms within Company A in Qld. This is clear evidence that it is possible for a single genotype to be dominant within a company. The dominance of genotype 277 in Company A was accompanied by a recognition that type 277 did occur in other Qld companies, but at a very low level. This finding for Company A in Qld is similar to the situation in New Zealand. Mullner *et al*. (2010a) showed a dominant human-associated *C. jejuni* genotype was dominant in one poultry supplier and absent in other suppliers.

Unlike the New Zealand study that examined poultry products, our study focused on caecal samples. This means no conclusions can be drawn about genotypes of *C. jejuni* present on poultry products. However, it is clear from our study that a company can have a dominant genotype of *C. jejuni* that persists over time (for at least one year) and across different flocks on different farms.

The emergence of the unique set of unresolved isolates in NSW during the national survey is of particular interest. These isolates are grouped together because they tested positive in both the *mapA* assay for *C. jejuni* and the *ceuE* assay for *C. coli*. The isolates also amplified in only one of the six allele specific assays in the MLST SNP HRM typing for *C. jejuni*. These results indicate that they are *C. coli* isolates that have acquired the *C. jejuni* specific *mapA* gene by lateral transfer and potentially other genes as well. This phenomenon of interspecies horizontal gene transfer has been reported previously. Schouls *et al*. (2003) described *C. coli* isolates that had received the *pgm* gene or a gene fragment from *C. jejuni* by interspecies horizontal transfer.

The unresolved isolates were persistent across time in NSW, representing 37% in the national survey in year one and 43% in the NSW survey in year two. They were also present in NSW in all four companies and in the free range flocks from Company B. In comparison, in the Qld regional survey, these unresolved isolates appeared in year three of the study and were specific to Company C. This was in both conventional and free range flocks, going from zero presence in year one to one of the dominant types present in year three. While the unresolved, or NT-1, isolates are being held together as one group, future typing methods may provide or elucidate other genetic diversity within the group that our current method cannot.

If we take a closer look at free range flocks that were included in the study, there were two genotypes that were unique. One type (335) was observed in Qld free range flocks as part of the national survey and one type (111) was observed in Company B in year two during the regional survey in NSW. All other genotypes that were observed in free range flocks have been observed in conventional flocks as well. The fact that the free range flocks shared common genotypes with conventionally raised flocks suggests both groups are exposed to similar sources of *Campylobacter*. As the free range flocks have a direct exposure to the external environment (a major source of *Campylobacter*), it would seem the external environment is also the major source of *Campylobacter* for conventionally raised birds. Several other studies (van de Giessen *et al*. 1996; Hald *et al*. 2000) have also identified the external environment as the major source of *Campylobacter* for broiler flocks.

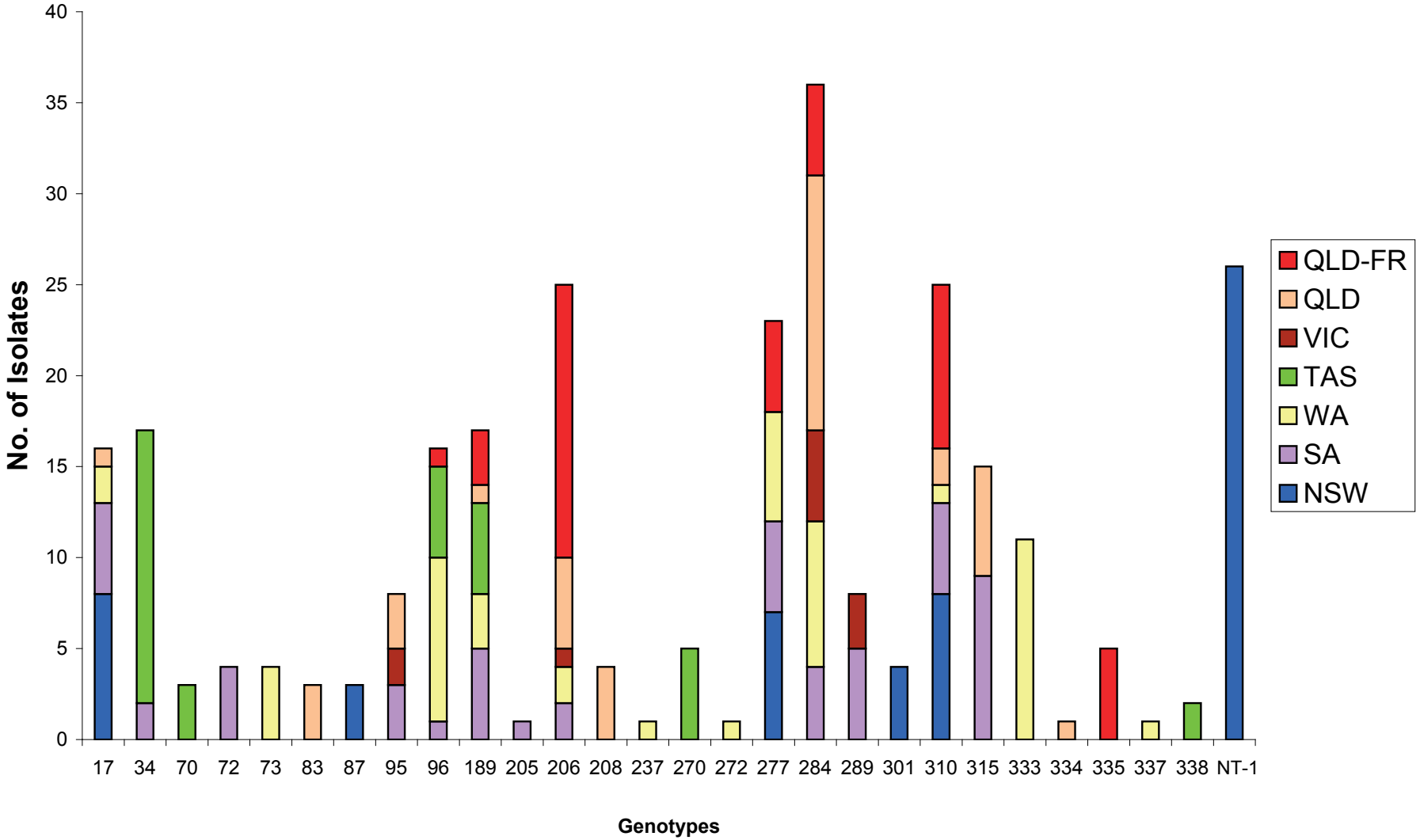

Figure 7.4 Comparison of genotypes found in the national survey

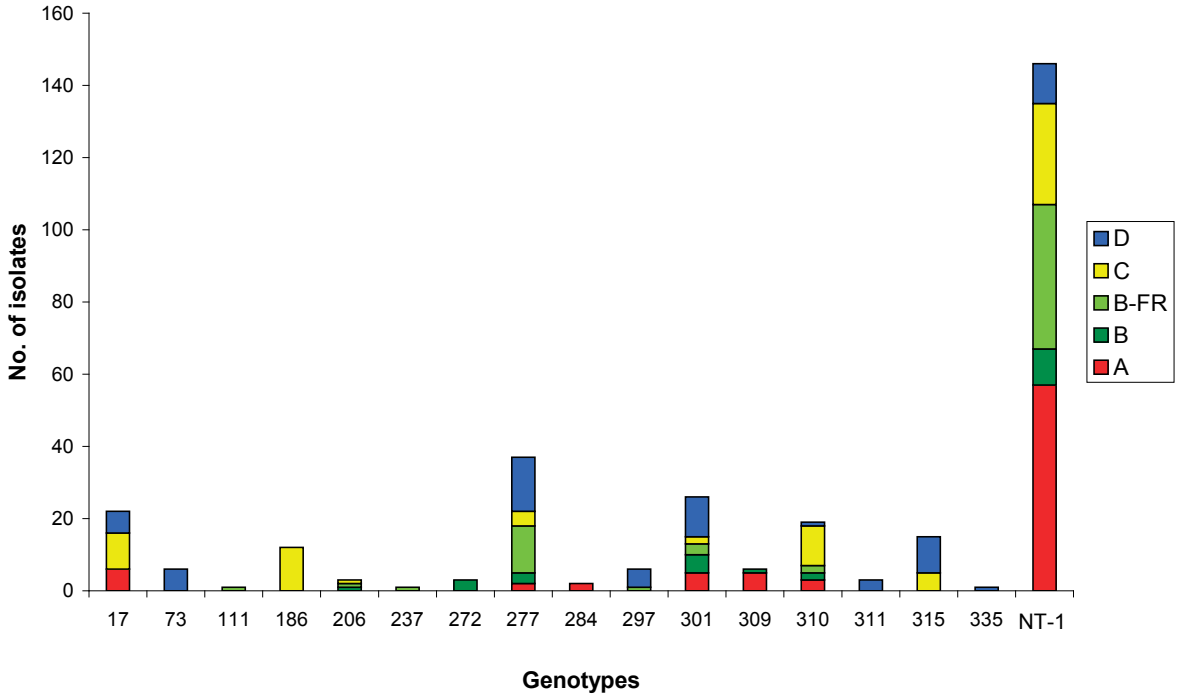

Figure 7.5 Comparison of genotypes in the NSW regional survey

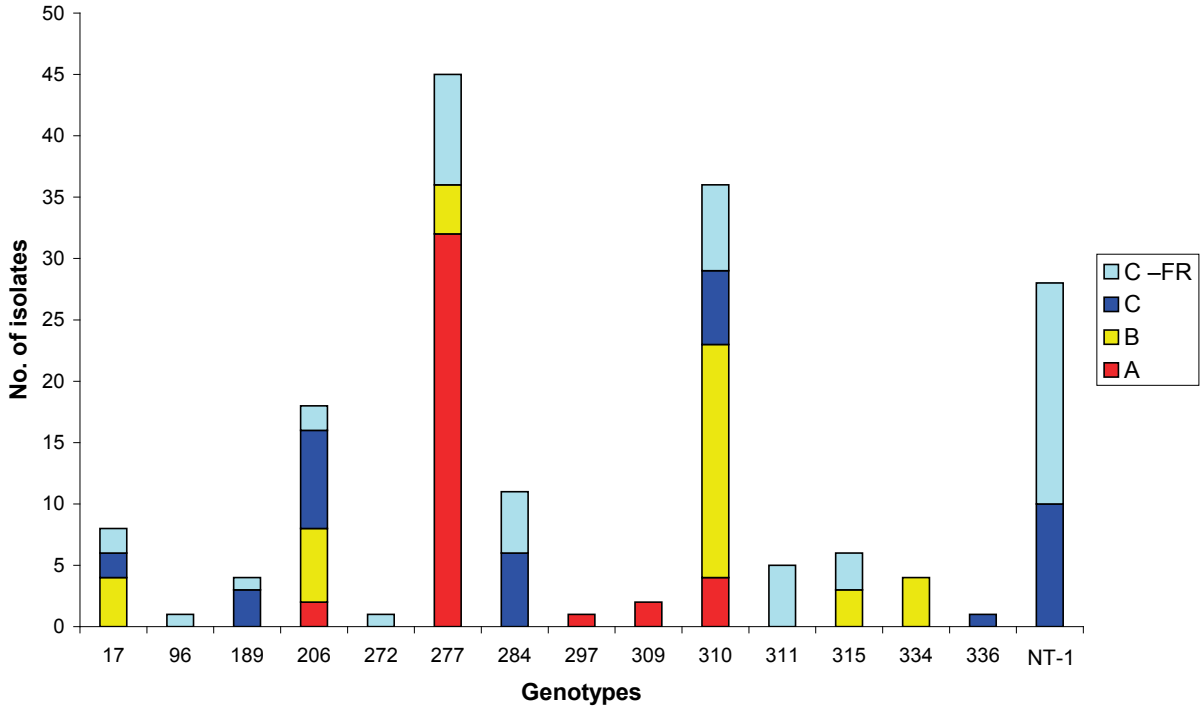

Figure 7.6 Comparison of genotypes in the QLD regional survey

In the NSW multi-year study, five out of 16 genotypes persisted across year one and two and two types (277, 310) were dominant in both years. Also, the unresolved isolates were present across both years and were the dominant type during this period (as shown in Figure 7.7 below). Similar results were seen in the Qld multi-year study from years one and three of the project (as shown in Figure 7.8 below).

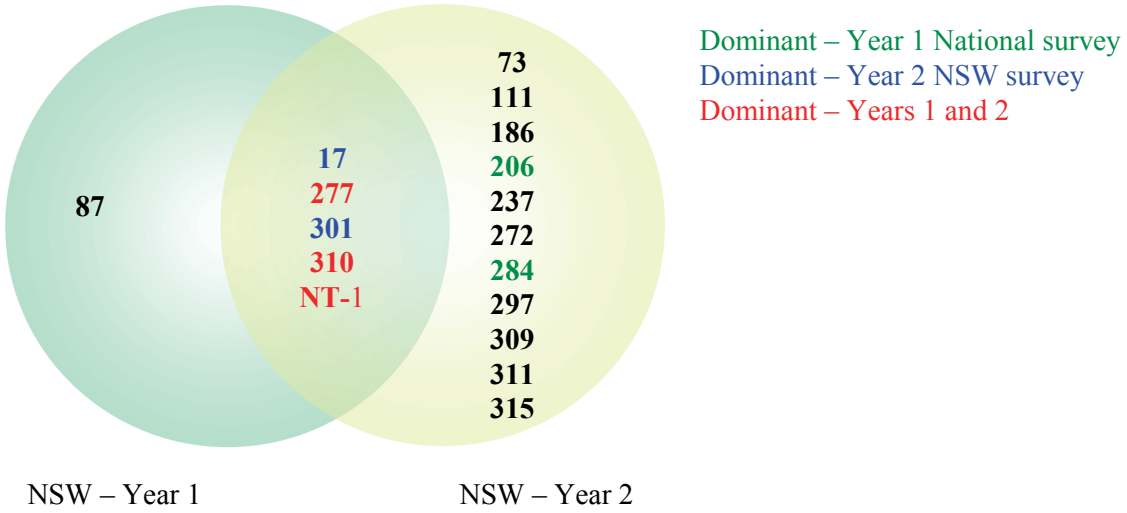

Figure 7.7 Venn diagram showing genotypes found in NSW during years one and two of the study

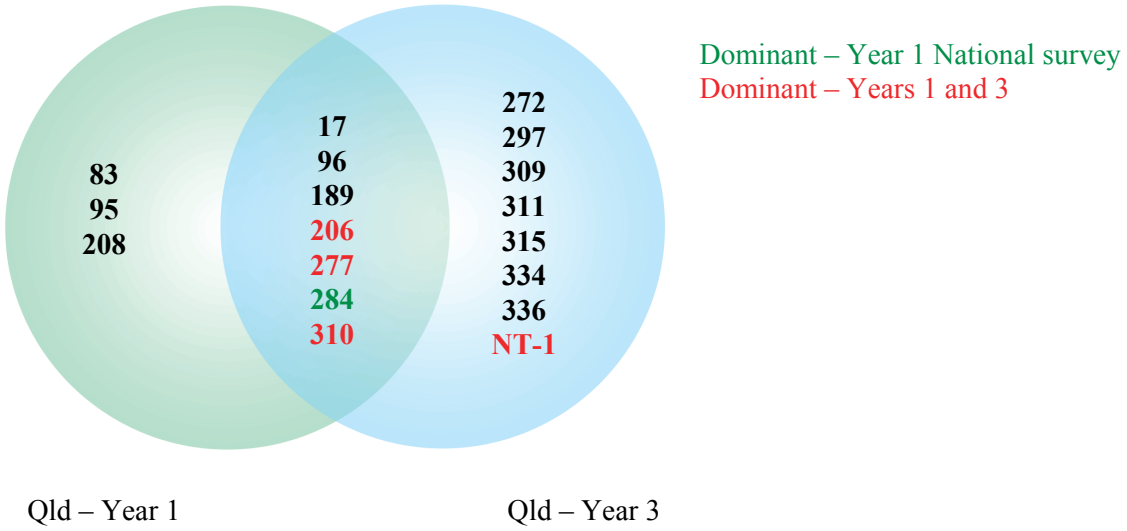

Figure 7.8 Venn diagram showing genotypes found in Qld during years one and three of the study

In the conventional poultry flocks, there were five out of nine types that persisted over the three years of the project and two types (206, 310) that were dominant in both years. Results for the free range flocks in Qld showed six out of 10 types persisted over the three years and three types (206, 277, 310) were dominant in both years. When comparing these results, it is clear that more genotypes persisted across time in Qld free range flocks (six compared with five in NSW and five in Qld conventional). There were also more dominant genotypes that persisted across time in Qld free range and these types persisted for a longer time period (three years) than the NSW survey (two years).

While the NSW Health Department *Campylobacter* culture collection was no longer viable, much valuable information has been gained from the collection. The power of molecular identification and typing methods has allowed this information to be gathered even from a non-viable culture collection.

Speciation results for the NSW human *Campylobacter* isolates were 84% *C. jejuni* and 16% *C. coli.* These results are comparable with other studies in the literature. Two studies in the UK have reported 91% *C. jejuni* and 9% *C. coli* (Sopwith *et al.* 2009) and 93% *C. jejuni* and 7% *C. coli* (Gillespie *et al*. 2002). Similarly, in New Zealand, Mullner *et al.* (2010b) in 2010 reported 88% *C. jejuni*.

Table 6.1 indicates almost 60% of the NSW human isolates belong to genotypes that we have detected in chickens from a major regional supply source (Mangrove Mountain) for the Sydney market. The distribution of chicken and human genotypes in NSW is presented below in Figure 7.9. This level of possible connection between poultry and humans is also typical of that reported in other studies. For example, a big study in Scotland attributed 76% of campylobacteriosis cases to chicken sources using MLST (Sheppard *et al.* 2009b).

Type 310 is an example of a possible connection between poultry and humans. This type was the most common genotype of *C. jejuni* in chicken in NSW in year one and two of the project. This genotype was also the most common genotype in humans.

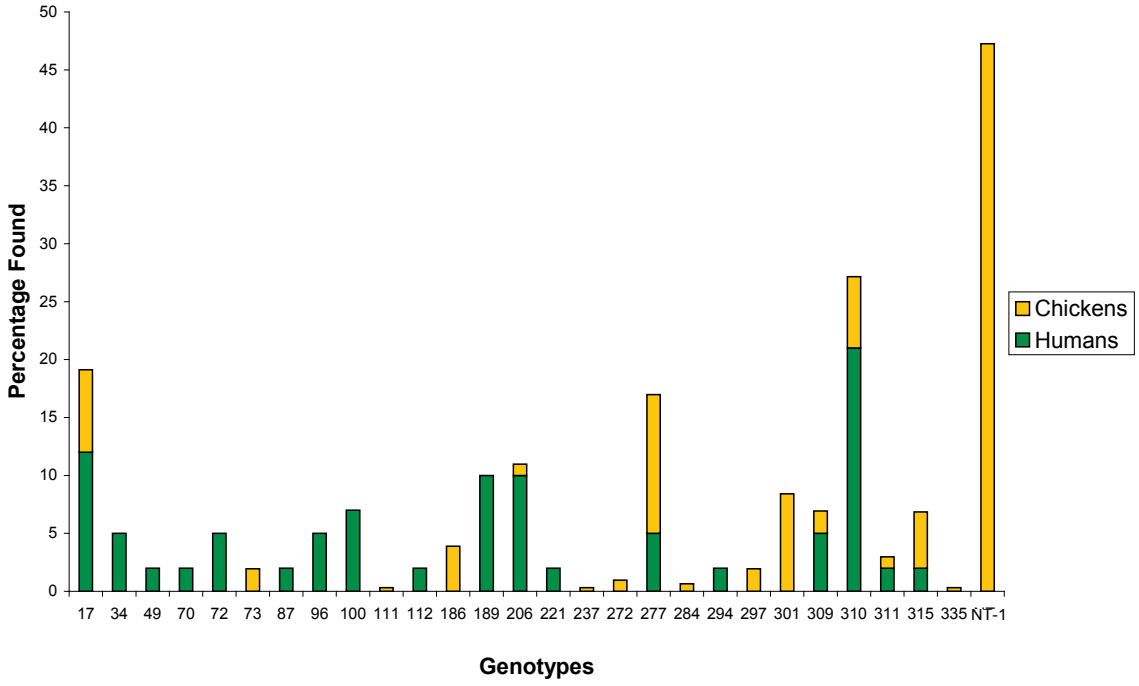

Figure 7.9 Distribution of genotypes for NSW chicken and human isolates in year two of survey

The level of information that can be gained from the NSW human isolates has not been fully exploited in our work. Our access to this culture collection was only possible during the final months of the project when time and budgets were limited. The culture collection that was forwarded consisted of 246 (non-viable) isolates, but we have only had time and resources to work on 50 isolates. It is possible that other insights and more substantive conclusions could be drawn if the remaining isolates were examined.

Overall, the emerging picture about genotypes of *C. jejuni* is:

- no one dominant genotype was found across the national company studied
- a small number of genotypes were common across flocks in different states, suggesting possible common environmental sources of *C. jejuni*
- shared genotypes across different companies in a geographical region also suggests common shared environmental source*s*
- some genotypes can persist across time
- some genotypes do not persist across time
- one company showed a marked dominance of a single genotype of *C. jejuni* (from more than one year of sampling and across multiple farms)
- one particular genotype, NT-1 (possibly *C. coli*), is an abundant genotype in a NSW region across companies and across multiple years
- the same unresolved, or NT-1, genotype (possibly *C. coli*) also emerged in a Qld region but only in one company and is absent in other companies operating in the same region
- free range flocks essentially share the same genotypes as those seen in conventionally raised flocks.

Implications

This study has several important implications:

- there is evidence that a company can have a dominant genotype of *C. jejuni* that persists across one year at least and across multiple farms
- the domination of a genotype is not the normal situation, as all other companies (three in Qld and four in NSW) did not show this effect
- there is evidence of a regional influence that results in companies within a region showing shared genotypes of *C. jejuni* in poultry at slaughter
- there is mixed evidence that some genotypes persist across multiple years and other genotypes do not
- free range flocks show a similar range of genotypes of *C. jejuni* as those detected in conventional flocks. This suggests there is no unique risk, in terms of genotypes associated with *C. jejuni* colonisation, associated with free range production systems
- there is dominance in NSW of a novel genotype (that is possibly *C. coli*) across companies in one production region. This genotype has persisted in that region and has now also emerged in one company in Qld.

Recommendations

This report provides new information important to the chicken industry and food safety professionals. This includes an understanding of the distribution of genotypes of *C. jejuni* throughout the Australian chicken meat industry and the potential influences of region, company and time on the presence of genotypes. Formal publications and conference papers are planned to ensure this information reaches relevant target groups (food safety professionals and the chicken meat industry).

Several recommendations for food safety professionals, regulators and the industry research body have arisen from this study:

- MLST SNP HRM should be considered as a front line method when typing *C. jejuni*
- consideration should be given to regular genotype monitoring to detect occurrences of a single genotype becoming dominant within a company
- further studies are required to enable confident identification (at the species level) and understanding of the public health implications of the unresolved, or NT-1, genotype that is now dominant in NSW and emerging in Qld.

Appendix 1: Collection of Caecal Samples

Sample collection

- Four farms are to be sampled collecting five samples from each farm.
- Older flocks are to be selected, second pick up birds > 40 days old.
- Five caeca from individual birds to be collected – not bunched together but randomly selected from throughout the flock.
- Total of 20 samples from four different farms.

Equipment required:

- Box of sterile disposable gloves
- Sharp scissors
- 70% ethanol for sterilisation
- Portable flame for sterilisation
- Tissues
- Discard container

Procedure:

1. Wearing a pair of sterile gloves, remove a randomly selected carcass from the production line.
2. Open carcass and remove the intestines, identifying the caeca.
3. Discard gloves and replace with a new pair of sterile gloves.
4. Sterilise scissors by dipping in/spraying with 70% ethanol and flame (if possible).
5. Snip off caeca into a sterile collection bag and seal.
6. Re-sterilise scissors using tissues and 70% ethanol and flame (if possible).
7. Discard gloves and waste.
8. Repeat procedure to collect five samples from the one farm.
9. Record details of samples collected on the data sheet provided and transport with samples.
10. Hold samples at 4ºC and transport as soon as possible.

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Campylobacter genotypes in chickens –National and regional influences

By Jillian Templeton

Pub. No. 14/032

This report describes the validation and application of a DNA-based typing scheme to more than 650 *Campylobacter* isolates, originating predominantly from poultry sourced across Australia.

Food-borne illness caused by *Campylobacter* continues to be the most frequently reported notifiable infection in humans in Australia, with annual rates of infection about double those for *Salmonella* spp.

This report is targeted at Australian chicken meat processing companies, food safety professionals and food safety regulators.

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