

# Phenotypic characterisation of Australian sheep and cattle isolates of *Mannheimia haemolytica*, *Mannheimia granulomatis* and *Mannheimia varigena*

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**Objective** To perform a comprehensive phenotypic characterisation of 35 isolates of bacteria previously identified as haemolytic *Pasteurella-Actinobacillus* and obtained from cattle and sheep.

**Design** The 35 isolates that had been obtained from Australian animals, 30 from cattle and five from sheep, were compared with reference strains of the five recognised species of the genus *Mannheimia* – *M haemolytica*, *M glucosida*, *M granulomatis*, *M ruminalis* and *M varigena*.

**Results** Thirty-four of the isolates could be confidently assigned to three species of the genus *Mannheimia*. Twenty-nine were *M haemolytica*, with 25 being isolated from cattle and four from sheep. All but three of the bovine *M haemolytica* were isolated from pneumonic lungs. Of the three remaining bovine *M haemolytica* isolates, one was obtained in pure culture from a bovine milk sample and the other two as part of a mixed flora associated with a middle ear infection of a calf suffering mucosal disease. Of the four ovine *M haemolytica* isolates, two were isolated in pure culture from milk and two, also in pure culture, from pneumonic lungs. Three bovine isolates were identified as *M granulomatis* – one from a tongue abscess, one from a jaw abscess and one from a lung showing suppurative bronchopneumonia. Two bovine isolates were identified as *M varigena* – one coming from an udder and the other from a spleen. The available diagnostic records provided no information on whether these isolates were associated with a disease process. The remaining isolate was obtained from an ovine tongue abscess and could not be assigned to a recognised species within the genus *Mannheimia*.

**Conclusion** The study represents the first time that *M haemolytica*, *M granulomatis* and *M varigena* have been recognised as being present in cattle and sheep in Australia. Veterinary laboratories that encounter *Pasteurella-Actinobacillus*-like organisms from cattle and sheep should attempt as complete a characterisation as possible to help improve our knowledge of the disease potential of these organisms.

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Members of the genus *Pasteurella* are well recognised pathogens of many species of animals.<sup>1</sup> The organism until recently known as [*Pasteurella*] *haemolytica* (the brackets indicate that this taxon was excluded from the genus *Pasteurella sensu stricto* in 1985<sup>2</sup>) has long been associated with

respiratory disease in cattle<sup>3</sup> and both respiratory disease and septicaemia in sheep.<sup>4</sup> Two biotypes of [*P*] *haemolytica* have been traditionally recognised – biotype A, consisting of isolates that ferment L-arabinose, and biotype T, consisting of isolates that ferment trehalose.<sup>5,6</sup> Isolates of biotype A of [*P*] *haemolytica* are associated with respiratory disease of cattle and sheep and septicaemia of young lambs while biotype T isolates have been associated with septicaemia of young adult sheep.<sup>4</sup>

Despite a general acceptance that isolates of biotype T [*P*] *haemolytica* are not closely affiliated with the genus *Pasteurella sensu stricto*, these organisms have been classified as [*P*] *trehalosi*.<sup>7</sup> In terms of serovars, [*P*] *trehalosi* contains [*P*] *haemolytica* serovars 3, 4, 10 and 15.<sup>7</sup>

A new genus, *Mannheimia*, has been recently proposed for the biotype A isolates of [*P*] *haemolytica*.<sup>8</sup> The genus of *Mannheimia* has five species – *M haemolytica*, *M glucosida*, *M granulomatis*, *M ruminalis* and *M varigena*.<sup>8</sup> The bulk of what were once regarded as biotype A [*P*] *haemolytica*, specifically [*P*] *haemolytica* serovars 1,2,5,6,7,8,9,12,13,14 and 16, are now recognised as *M haemolytica*. The isolates once recognised as [*P*] *haemolytica* serovar 11 have been reclassified as *M glucosida*.<sup>8</sup> It should be noted that serotyping does not always result in a clear, species-specific identification.<sup>9</sup>

In the Australian context, there is considerable evidence that [*P*] *haemolytica* is a routine isolate from pneumonic bovine lungs.<sup>10-13</sup> Taylor<sup>14</sup> has reported on an outbreak of fibrinous pneumonia in 331 recently weaned beef calves on two southern Queensland properties. While no [*P*] *haemolytica* was isolated from this outbreak, Taylor<sup>14</sup> concluded that the clinical and necropsy findings suggested that the cause was [*P*] *haemolytica*. Mastitis in dairy cattle has also been associated with [*P*] *haemolytica*.<sup>13</sup> Recently, the presence of a novel taxon of the genus *Mannheimia*, of uncertain pathogenicity, has been reported from nasal swabs of feedlot cattle.<sup>15</sup>

In Australian sheep, there have been reports of pneumonia, mastitis<sup>13,16</sup> and septicaemia<sup>17</sup> associated with [*P*] *haemolytica*.

We have re-examined a collection of bacteria previously identified as *Pasteurella-Actinobacillus* isolates from cattle and sheep. These isolates were all identified during routine disease investigations performed at the Animal Research Institute and the Toowoomba Veterinary Laboratory between 1963 and 1997. The isolates were obtained from 29 cattle (30 isolates) and five sheep (five isolates).

## Materials and methods

### Bacteria

Thirty five isolates were studied. The available details on these isolates including the original identification is provided in Table 1. The reference strains that were used in this study were *M haemolytica* NCTC 9380, *M granulomatis* ATCC 49244, *M glucosida* CCUG 38457, *M ruminalis* CCUG 38470 and *M*

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Table 1. Details of the cultures examined in this study.

Number of Cultures	Original identification	Correct species	Animal species	Tissue	Other pathogens	Diagnosis
1	<i>P haemolytica</i>	<i>M granulomatis</i>	Bovine	Lung	None	Enteritis (lung showed suppurative bronchopneumonia)
1	<i>Actinobacillus</i> spp <sup>a</sup>	<i>M granulomatis</i>	Bovine	Jaw abscess	None	Lumpy jaw
1	<i>Actinobacillus</i> spp <sup>b</sup>	<i>M granulomatis</i>	Bovine	Tongue	None	Actinobacillosis tongue
7	<i>P haemolytica</i>	<i>M haemolytica</i>	Bovine	Lung	None	Bronchopneumonia, pasteurellosis
6	<i>P haemolytica</i>	<i>M haemolytica</i>	Bovine	Lung	none	Pleuropneumonia, pasteurellosis
2	<i>P haemolytica</i>	<i>M haemolytica</i>	Bovine	Lung	<i>P multocida</i>	Pleuropneumonia, pasteurellosis
1	<i>P haemolytica</i>	<i>M haemolytica</i>	Bovine	Lung	Infectious bovine rhinotracheitis virus	Bronchopneumonia, pasteurellosis
1	<i>P haemolytica</i>	<i>M. haemolytica</i>	Bovine	Lung	Mucosal disease	Encephalitis (Lung showed severe fibrinopurulent bronchopneumonia)
3	<i>P haemolytica</i>	<i>M haemolytica</i>	Bovine	Lung	None	Pasteurellosis
2	<i>P haemolytica</i>	<i>M haemolytica</i>	Bovine	Middle ear	Mucosal disease	Mucosal disease
1	<i>Actinobacillus</i> spp.	<i>M haemolytica</i>	Bovine	Lung	None	Pneumonia
1	<i>Pasteurella</i> spp.	<i>M haemolytica</i>	Bovine	Lung	None	Pneumonia
1	<i>Actinobacillus</i> spp.	<i>M haemolytica</i>	Bovine	Milk	None	Mastitis
1	<i>P mastitidis</i>	<i>M haemolytica</i>	Ovine	Milk	None	Mastitis
1	<i>Pasteurella</i> spp.	<i>M haemolytica</i>	Ovine	Lung	None	Pneumonia
1	<i>Actinobacillus lignieresii</i>	<i>M haemolytica</i>	Ovine	Milk	None	Mastitis
1	<i>Actinobacillus lignieresii</i>	<i>M haemolytica</i>	Ovine	Lung	None	Pneumonia
1	<i>Actinobacillus</i> spp <sup>a</sup>	<i>M varigena</i>	Bovine	Udder	None	Unknown
1	<i>Actinobacillus</i> spp	<i>M varigena</i>	Bovine	Spleen	None	Kidney fibrosis
1	<i>Actinobacillus</i> spp <sup>a</sup> (V4619)	<i>Mannheimia</i> spp.	Ovine	Tongue abscess	None	Unknown

<sup>a</sup>Indicates strains previously described by Angen et al<sup>18</sup>

<sup>b</sup>Indicates strain previously described by Angen et al<sup>9</sup>

*varigena* CCUG 38462. All of these are type strains for their respective species.

#### Phenotypic characterisation

All the field isolates as well as the reference strains were subjected to a full phenotypic characterisation as previously described.<sup>18,19</sup>

#### Case histories

The available case histories were reviewed. In many instances, the details of the histopathological findings were no longer available. As well, for many of the cases, only a brief summary of the original findings could be located.

## Results

All 35 field isolates were Gram negative, catalase and cytochrome oxidase positive, non-motile rods that fermented glucose without gas production. The isolates had no requirements for X- or V-factors in vitro, were unable to utilise citrate, did not produce any pigment, could not grow in potassium cyanide and were negative in the methyl red and Voges-Proskauer tests. All the isolates could reduce nitrate without gas formation but lacked the ability to hydrolyse arginine, decarboxylate lysine and could not hydrolyse gelatin, Tween 20 or Tween 80. None of the isolates could grow on MacConkey agar or deaminate phenylalanine but all showed phosphatase activity. All the isolates lacked urease activity. Negative reactions were also obtained in mucate (acid), malonate (base) and TSI (H<sub>2</sub>S)

tests. None of the isolates produced acid from meso-erythritol, adonitol, D(+) arabinol, xylitol, L(-) xylose, dulcitol, D(+) fucose, D(+) mannose, L(-) sorbose, D(+) melibiose, trehalose, D(+) melezitose, D(+) glycogen, inulin, D(+) turanose and β-N-CH<sub>3</sub>-glucosamide. All the isolates produced acid from D(-) ribose, D(-) mannitol D(-) fructose, D(+) galactose and sucrose. The isolates differed in a number of properties and the details of these distinguishing properties are given in Table 2, which also shows the properties of the type strains used in this study.

On the basis of the differential properties shown in Table 2, the field isolates were identified as *M haemolytica* (29 isolates), *M granulomatis* (three isolates) and *M varigena* (two isolates). The remaining isolate could not be assigned to any currently recognised taxon in the family *Pasteurellaceae*.

Table 1 provides a summary of the relevant information obtained from the review of the case histories. The bovine *M haemolytica* isolates were typically associated with bronchopneumonia and pleuropneumonia. In general, there were no other pathogens associated with these conditions. This lack of other pathogens needs to be interpreted cautiously because the isolates were obtained from routine disease investigations. In particular, it is possible that anaerobic pathogens may have been present and not detected. One bovine *M haemolytica* isolate was associated with mastitis. The four ovine *M haemolytica* isolates were associated with either mastitis (two isolates) or pneumonia (two isolates). The three *M granulomatis* isolates were obtained from cattle – two being associated with oral cavity abscesses and one with suppurative bronchopneumonia. The two *M varigena*

Table 2. Properties of Australian isolates of *Mannheimia* and those of the type strains of the five recognised species of the genus *Mannheimia*<sup>a</sup>

Property	<i>M. haemolytica</i>		<i>M. granulomatis</i>		<i>M. varigena</i>		<i>M. glucosida</i>	<i>M. ruminalis</i>	V4619
	NCTC 9380 <sup>T</sup>	Field (30) <sup>b</sup>	ATCC 49244 <sup>T</sup>	Field (3)	CCUG 38462 <sup>T</sup>	Field (2)	CCUG 38457 <sup>T</sup>	CCUG 38470 <sup>T</sup>	Field (1)
Haemolysis	w <sup>c</sup>	w	-	-	w	w	w	-	w
ODC	-	-	-	-	+	+	+	-	-
Indole	-	v [1] <sup>d</sup>	-	-	-	-	-	-	-
Acid from									
Glycerol	-	(+)	(+)	(+)	(+)	(+)	(+)	-	(+)
D-Arabinose	-	v [12]	w	v [2]	w	v [1]	w	(+)	(+)
L-Arabinose	-	-	-	-	+	+	-	-	+
Dextrin	+	+(+)	(+)	(+)	w	+	+	-	(+)
L-Fucose	+	v [28]	w	(+)	+	(+)	+	w	+
Glycosides <sup>d</sup>	-	-	+	+(+)	-	-	+	-	-
meso-Inositol	w	v [29]	w	-	(+)	(+)	(+)	-	-
Lactose	-	v [24]	w	(+)	-	(+)	(+)	(+)	(+)
Maltose	+	+	(+)	+(+)	+	+	+	-	+
L(+) rhamnose	-	-	-	-	+	(+)	-	-	-
Raffinose	(+)	v [29]	-	-	(+)	-	(+)	-	(+)
D-Sorbitol	+	+	+	+	-	-	+	-	-
D-Xylose	+	+	-	+	+	+	+	-	+
α-fucosidase	+	v [27]	-	-	+	-	+	-	-
β-galactosidase	-	v [27]	+	+	+	+	+	+	+
β-glucuronidase	+	v [4]	+	-	-	-	-	-	-
β-xylosidase	-	v [1]	-	-	-	-	+	-	-

<sup>a</sup>All isolates were Gram-negative, non-motile rods that fermented glucose without gas production. The isolates were oxidase and catalase positive, reduced nitrate to nitrite and could not grow on MacConkey agar. The isolates did not produce acid from trehalose and xylose but did produce acid from mannitol, galactose and sucrose. Further results are provided in the main text. The genus *Mannheimia* has been created from organisms that were previously termed biotype A [*P*] *haemolytica*.

<sup>b</sup>Number in round brackets is the number of field isolates tested

<sup>c</sup>ODC = ornithine decarboxylase; w = weak positive; + = positive within 1–2 days; (+) = positive within 14 days; v = variable; - = negative

<sup>d</sup>Number in square brackets is the number of isolates positive. Glycosides = cellobiose, β-glucosidase, aesculin, amygdalin, arbutin, gentiobiose and salicin. The result in the Table is the result for all of these substrates.

isolates were from cattle. One isolate was obtained from the spleen of a cow showing kidney fibrosis. The other isolate was obtained from an udder but the available records provided no evidence on whether the isolate was involved in a pathological process. The remaining isolate, which could not be assigned to any of the five recognised species of the genus *Mannheimia*, was obtained from an ovine tongue abscess.

## Discussion

This study has shown the problems confronting diagnostic laboratories attempting to identify members of the family *Pasteurellaceae*. Of the 29 isolates shown in this study to be *M haemolytica*, 23 were originally correctly identified – albeit in the old terminology of either [*P*] *haemolytica* (22 isolates) or [*P*] *mastitidis*. The remaining six isolates of *M haemolytica* were originally identified as *Actinobacillus lignieresii*, *Actinobacillus* spp or *Pasteurella* spp. Our finding that some of the original identifications were incorrect should not be seen as a result of any shortcomings by the original workers. The misidentifications are clear evidence of the fact that identification of bacteria that belong in the family *Pasteurellaceae* is a difficult task that requires considerable effort. The current study has the advantage of an extensive bank of new knowledge and additional phenotypic tests that make correct identifications much easier. A similar conclusion on the difficulties of identifying *A equuli* and relatives has been reported already.<sup>20</sup>

Laboratories that have a regular workload of identifying *Mannheimia*-like isolates should consider obtaining reference strains to ensure that the identification methods used in the laboratory are acceptable. Since many diagnostic laboratories in

Australia are moving to formal accreditation, this use of reference strains will become part of the quality control operations of these laboratories. Research into the development and validation of molecular-based identification methods would also be of great value. Fully validated molecular methods offer considerable advantages over the complex and extended phenotypic identification schemes necessary for confident identification. Already, there are molecular identification methods for *Haemophilus paragallinarum*<sup>21</sup> and *Pasteurella multocida*,<sup>22–24</sup> which are other members of the *Pasteurellaceae*.

The results of this study have shown that, like the situation in overseas countries,<sup>8</sup> the main species of the genus *Mannheimia* associated with pneumonia and mastitis in cattle and sheep is *M haemolytica*. *M haemolytica* corresponds to [*P*] *haemolytica* serovars 1, 2, 5–9, 12–14 and 16.<sup>8</sup> Currently, serotyping for *M haemolytica* is not available in Australia. This is a major limitation that prevents a more definitive understanding of bovine pneumonia – an economically important disease, particularly of feedlot cattle.

Four of the isolates included in this study, marked in Table 1, have been included in the prior studies of Angen et al.<sup>8,18</sup> However, only one was included in the final publication on reclassification of the [*P*] *haemolytica* complex.<sup>8</sup> Due to recent changes in the terminology of these organisms and the minor role these four isolates played in these complex prior taxonomic studies, we believe that veterinary microbiologists working in Australian veterinary laboratories may not realise that isolates of the new genus *Mannheimia* have been confirmed as being present in Australia. Hence, we have included these four previously studied isolates in the current report.

The results of the current study, combined with the prior studies<sup>8,18</sup> have demonstrated the presence in Australian cattle of one of the more unusual species of the genus *Mannheimia* – *M granulomatis*. The three Australian isolates of *M granulomatis* appear to be the only reported cattle isolates of this species apart from the isolates reported from Brazil.<sup>25,26</sup> The Brazilian bovine isolates have been associated with a progressive fibro-granulomatous panniculitis in which it has been suggested that biting insects may play a role.<sup>25</sup> In a detailed histopathological study of both natural cases as well as experimental cases, Riet-Correra et al<sup>25</sup> noted the presence of small granulomas with rosettes in the centre. Riet-Correra et al<sup>25</sup> noted that these rosettes were very similar to those seen in actinobacillosis. The retrospective nature of our study makes it difficult for any conclusive statements to be made about the pathogenic role of the *M granulomatis* isolates from lumpy jaw and wooden tongue cases. In particular, we cannot rule out the possibility that other agents, including anaerobes, were present and may have had a role in the disease process. Nevertheless, our results indicate that laboratories culturing cases of lumpy jaw and wooden tongue should perform the level of phenotypic characterisation necessary to confidently identify any *Pasteurella*-like organisms isolated.

Two of the Australian isolates were first obtained in the 1960s, considerably predating the Brazilian description of panniculitis. These 1960 Australian isolates were recognised as being different from any known organism of the time, hence they were termed *Actinobacillus* species. *M granulomatis* can be readily separated from both *M haemolytica* and *A lignieresii* by phenotypic characterisation, the latter organism being the species normally associated with granulomatous lesions of the bovine tongue. Veterinary laboratories isolating an *Actinobacillus*-like organism from these types of oral lesions should attempt identification to the species level.

One of our isolates of *M granulomatis* was obtained in pure culture from a bovine lung with suppurative bronchopneumonia. This appears to be the first time that *M granulomatis* has been reported in a situation strongly suggesting a role in a pathogenic process of the bovine lung. In contrast, there is a strong association with bronchopneumonia for the leporine isolates of *M granulomatis*.<sup>19,27</sup> As our isolate was originally identified as [*P*] *haemolytica*, it is possible that other evidence of the association between bovine bronchopneumonia and *M granulomatis* has been missed. It is important that laboratories investigating bovine respiratory disease attempt as complete an identification of bacterial isolates as possible. Over time, this level of identification should result in an accumulation of evidence that will help clarify the role of *M granulomatis* in bovine bronchopneumonia.

Two of the isolates in this study were identified as *M varigena*. One of these isolates has been characterised previously as [*P*] *haemolytica* biogroup 6.<sup>18</sup> *M varigena* isolates form two biovars – biovar 1 are ornithine decarboxylase positive and are typically isolated from cattle whereas biovar 2 are ornithine decarboxylase negative and are typically isolated from pigs.<sup>8</sup> Bovine isolates of *M varigena* have been associated with disease conditions such as pneumonia, mastitis and septicaemia as well as being isolated from non-sterile sites such as the rumen, the oral cavity and the intestine.<sup>8</sup> The two Australian isolates do not have a detailed available history, although the spleen isolate does not seem to have had a role in any pathogenic process. Careful speciation of [*P*] *haemolytica*-like isolates into the new *Mannheimia* species is required before any overall assessment

can be made of the role of *M varigena* in bovine disease in Australia.

In summary, this study has confirmed the presence of three of the new species of the genus *Mannheimia* in Australian cattle and sheep – *M haemolytica*, *M granulomatis* and *M varigena*. The detailed identification table provided in this study (Table 2) should assist laboratories in this task of extended characterisation.

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## Comparison of infectivity of *Eimeria tenella* oocysts maintained at 4, 12 or 28°C for up to 10 months

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Coccidiosis is an economically important protozoan disease of poultry caused by members of the parasitic genus *Eimeria* (Coccidia: Eimeriidae).<sup>1</sup> Current methods for coccidiosis control in the Australian poultry industry depend on incorporation of anticoccidial agents into feed or water. The major drawbacks of chemical control include the growing problem of drug-resistance and the increasingly complex control programs required. An alternative method of coccidiosis control, which avoids the problems associated with drug resistance, is use of live attenuated vaccines either alone or in conjunction with an integrated chemical control program.

A requirement for both commercial production and distribution of live vaccines is an efficient storage method that maintains the infectivity of vaccine batches for an extended period. A long storage time is desirable as this allows larger batches to be produced with a reduction in costs per dose. The currently accepted method for storing *Eimeria* oocysts is to maintain the parasites at 4°C in 2% potassium dichromate or in sterile media.<sup>2-6</sup> There are few published reports<sup>7</sup> examining the rate of decline of infectivity of poultry *Eimeria* oocysts stored over time or comparing different storage temperatures. The aim of this experiment was to determine which of 4, 12 or 28°C is the best storage temperature for *Eimeria tenella* oocysts.

Male Webster strain White Leghorn chickens were used for all experimental work. They were reared until 4 weeks old under *Eimeria*-free conditions in positive pressure isolators supplied with HEPA filtered air.

During infectivity trials, birds were housed in suspended wire cages in a controlled environment room maintained at 25°C with light from 0600 h to 2200 h daily. Feed and water were available continuously. Feed was custom formulated without coccidiostats, heat treated during pelleting and stored at -20°C

for a minimum of 5 days to ensure freedom from viable *Eimeria*. All equipment used was sterilised by either autoclaving or heat treatment at 90°C for 4 h.<sup>8</sup> Faeces were collected in sterilised trays suspended under each cage.

At the start of the experiment, one pooled batch of sporulated oocysts was produced from faeces collected from six birds inoculated with a precocious vaccine strain of *E tenella* (Darryll strain). Two days after sporulation, 180 vials (5 mL) were prepared each containing 500 oocysts in 1 mL aliquots of 2% potassium dichromate solution. The contents of the vials were either immediately inoculated by gavage into chickens (time zero) or used in four infectivity trials involving storage for 2, 4, 6 or 10 months at 4 (commercial refrigerator, range 2.5 - 7°C), 12 or 28°C (laboratory incubators, range ± 1°C) (Table 1).

The design for measuring oocyst production from oocysts inoculated at time zero was 12 replicate cages. The experimental design of each of the four infectivity trials for stored oocysts was a randomised blocks layout of 12 cages, with four replicate cages for each of the three storage temperature treatments (4, 12 or 28°C). The experimental unit was a cage of three birds.

**Table 1 Mean oocyst production per bird after inoculation with 500 *E tenella* oocysts, either freshly sporulated or stored for various times at three different temperatures.**

Storage temperature °C	Storage time (months)				
	0	2	4	6	10
Nil Storage	134.2 (24.1)				
4		141.9 <sup>AB</sup> (28.6)	79.5 <sup>A</sup> (5.0)	58.8 <sup>B</sup> (2.0)	0 <sup>B</sup> (0)
12		170.5 <sup>A</sup> (49.6)	104.1 <sup>A</sup> (11.3)	121.1 <sup>A</sup> (17.8)	78.4 <sup>A</sup> (4.8)
28		110.0 <sup>B</sup> (13.3)	0 <sup>B</sup> (0)	0 <sup>C</sup> (0)	ND

Mean oocyst production is cube root transformed; oocyst numbers ( $\times 10^{-5}$ ) are given as back transformed means in parenthesis.

Means within columns followed by different superscript letters are significantly different at the 5% level.

LSD (two-tailed *t* test, *P* = 0.05) = 32.7 for comparing storage temperature means, at the same storage time only, on the transformed scale.

LSD (one-tailed *t* test, *P* = 0.05) = 27.2 for comparisons involving storage temperature treatment means versus zero oocyst production, at the same storage time only, on the transformed scale.

ND not done.