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Typing of *Pasteurella multocida*

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Foreword

Fowl cholera is caused by the bacterium *Pasteurella multocida* and remains one of the major bacterial diseases of poultry. Our ability to control this disease has not advanced much since the pioneering work of Louis Pasteur who developed a vaccine in the late 1890s. This work by Pasteur represented the first time that a vaccine was developed for a bacterial disease of humans or animals. Over one hundred years later, the Australian poultry industry still uses fowl cholera vaccines that are very similar to that produced by Pasteur. One area where there has been significant advances is our ability to type isolates of *P. multocida*. With an increased ability to type a pathogen, there is potential to better understand disease outbreaks (where, how and why a disease agent enters a farm) and thus better chances at designing sustainable control programs. The importance of *P. multocida* as a pathogen of birds and many other species is such that the complete chromosome of this pathogen was recently sequenced. This availability of the chromosome sequence allows the application of cutting edge DNA typing methods.

The objective of the project was to establish a novel DNA-based typing scheme for *P. multocida*. The selected technology was Multi-locus Sequence Typing (MLST). The establishment of an MLST scheme for *P. multocida* will allow any isolate to be directly compared with any previous isolate already typed and thus will allow an understanding of the epidemiology of fowl cholera outbreaks. This improved understanding will lead to improved prevention and control programs for fowl cholera.

This publication describes the development of the first ever MLST scheme for *P. multocida*. The work involved the sequencing of seven genes in 66 strains of *P. multocida*. The DNA sequences were then used to create a dendogram to show the inter-relationships of the isolates. The data-base of gene sequences has also been lodged at the international MLST Website - http://www.mlst.net/. The ability of the newly established typing scheme to investigate fowl cholera outbreaks was evaluated. MLST typing was shown to be specific, sensitive and stable. Hence, MLST typing is now the ideal method for the investigation of fowl cholera outbreaks.

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

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Peter O'Brien
Managing Director
Rural Industries Research and Development Corporation

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As this report represents a significant portion of the M. Sc. thesis of Sounthi Subaaharan, the following acknowledgements from the M. Sc. thesis of Sounthi Subaaharan are reproduced:-

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Executive Summary

The aim of this project was to develop a multi-locus sequence typing (MLST) scheme for *Pasteurella multocida*, a facultative Gram-negative coccobacillus that causes a wide range of diseases in both wild and domestic animals (Adler *et al.*, 1996). Some serovars of *P. multocida* are the aetiologic agents of severe pasteurellosis conditions such as fowl cholera in poultry, haemorrhagic septicaemia in cattle and buffalo and atrophic rhinitis in pigs (Adler *et al.*, 1996). The contagious nature of fowl cholera, combined with high morbidity and mortality, has made the disease economically important where-ever poultry are raised (Glisson *et al.*, 2003).

From an epidemiological perspective, the process of strain typing is important for recognizing outbreaks of infection, detecting the cross-transmission of pathogens, determining the source of the infection, recognizing particularly virulent strains of organisms and monitoring vaccination programs. In essence, the information generated in typing studies is essential for identifying, tracking and controlling disease outbreaks.

Strain typing, whether for molecular epidemiology studies or population genetic studies, depends on accurate assessment of genetic diversity to address questions regarding genetic relatedness among individuals, population structure and phylogenetic relationships. Over time many different techniques have been developed to estimate genetic diversity, but no single technique is universally ideal; each available technique exhibits both advantages and disadvantages (Mueller & Wolfenbarger, 1999). Therefore the selection of the technique for application in a particular study is often on the basis of addressing the research question pursued and the solution needed, as well as on financial and technical availability (Mueller & Wolfenbarger, 1999).

MLST is emerging as an alternative typing which approaches almost an ideal technique (Maiden *et al.*, 1998). In basic terms, MLST is based on determining the DNA sequence changes in specific gene loci of a particular bacterial population. Specifically, MLST facilitates the discrimination of microbial isolates by comparing the sequences of housekeeping gene fragments. The main advantages of the MLST approach are defined characters (point mutations, insertions or deletions), which detect single base differences, and the information gathered is totally portable across laboratories (Maiden *et al.*, 1998). This means that a Web site can host the information bank – allowing laboratories around the world to deposit typing information AND directly compare their isolates with all other isolates in the bank (Maiden *et al.*, 1998). Hence, MLST is often suggested as the "gold standard" method for typing bacterial pathogens (Maiden *et al.*, 1998).

This project involved 63 isolates of *P. multocida* from Australian poultry – all associated with fowl cholera outbreaks. As well, three international reference strains, representing the three subspecies within *P. multocida* were included in the study. The complete sequence of seven different genes (all associated with various house-keeping activities within the bacterial cell) were determined. The sequences were then analysed using a specialised suite of software. This analysis showed that 39 different MLST Sequence Types (STs), representing 39 distinct allelic profiles, were present in the study. One particular ST (ST-2) was found to be the predominant ST (18.18%) in these isolates.

A dendogram showing the genetic relatedness of the isolates was produced. This dendogram showed a strong correlation with the dendogram created using the earlier phenotypic-based technology of multi-locus enzyme electrophoresis (MLEE). In particular, both MLST and MLEE have shown that there are two major sub-divisions within the species *P. multocida*. In accordance with the prior MLEE and ribotyping data, MLST has confirmed that a diverse range of *P. multocida* are associated with fowl cholera in Australia. A retrospective analysis confirmed that MLST has the power to be a useful typing tool – allowing outbreaks of fowl cholera to be followed and understood.

The major outcome of this project was the first web based MLST scheme for *P. multocida* – with this scheme being constructed on Australian poultry isolates. This is a significant advance – both in the Australian and international context. The scheme will enhance the management and control of fowl cholera outbreaks, both in Australia and around the world. The data-base generated in this study has been provided to the major MLST Web site http://www.mlst.net/.

The development of the MLST database and its placement on the central website serviced by the Oxford University means that laboratories around Australia and around the world can now directly compare isolates and build a truly international picture of the diversity within this important pathogen of poultry and other commercial livestock.

Introduction

The Disease

Fowl cholera is a common and widely distributed disease of poultry and is of major economic importance (Glisson *et al.*, 2003). The disease is caused by the bacterium *Pasteurella multocida* (Glisson *et al.*, 2003).

The disease can express itself in an acute or a chronic form. In the acute form, clinical signs are seen only in the few hours before death. Hence, if the birds are not observed in this short period, the first indication of the disease is often death. Chickens in the acute stage of the disease will show signs of fever, have ruffled feathers, have a mucus discharge from the mouth, suffer diarrhoea and show an increased breathing rate (Glisson *et al.*, 2003).

The chronic form of the disease can follow an acute stage or may be the only form of the disease present in a flock. The signs of this form of the disease are generally linked to localized infections. Wattles, sinuses, leg or wing joints, foot pads may become swollen. Swollen eyes (more correctly know as serous inflammation of the conjunctiva), twisted necks (more formally called torticollis), respiratory gurgles (rales) can occur. Chronically affected birds can progress to death, can recover or may stabilise (with the outcome of the latter two being a bird that is continuously infected and capable of spreading the disease to other birds) (Glisson *et al.*, 2003).

While most of the literature reports cases of fowl cholera in chickens, turkeys, ducks and geese, it is generally accepted that all types of birds are susceptible to the disease (Glisson *et al.*, 2003). In chickens, deaths occur most commonly in laying chickens as it is generally thought that chickens less than 16 weeks of age are generally resistant to clinical disease (Glisson *et al.*, 2003). Wild birds are susceptible to the disease – with deaths recorded in over 50 species to date (Glisson *et al.*, 2003). The outbreaks in wild birds can cause enormous mortalities e.g. over 40,000 waterfowl died in an outbreak in the San Franciso Bay area (Rosen & Bischoff, 1949).

In the Australian context, fowl cholera was first reported by Hart (1938) and is recognised as occurring in all Australian States (Beveridge & Hart, 1985). The disease has been recognised in meat breeder hens and chickens as well as laying hens (Grimes, 1975; Jackson *et al.*, 1972; Reid *et al.*, 1984). Hungerford (1968) described one of the most spectacular outbreaks of fowl cholera in which an infectious laryngotracheitis vaccine contaminated with *P. multocida* was administered to more than 90,000 chickens, with no deaths in the 20,000 vaccinated chickens less than 16 weeks of age but with severe mortality (90%) in the 70,000 vaccinated chickens over 16 weeks of age. In the only serological characterisation study performed in Australia, Ireland *et al.* (1989) reported that over 75% of 65 isolates of *P multocida* from Australian chickens were serovars 1, 3 or 3 cross-reacting with 4 (i.e. 3 X 4).

How does the disease enter a flock?

The chronically infected bird is regarded as the main source of infection (Blackall, 2003). A chronically infected bird can remain infected for life, with survivors of an acute outbreak having been shown to act as reservoirs of infection (Glisson *et al.*, 2003). In a USA study, birds held back from a flock that had suffered a fowl cholera outbreak provided a reservoir of infection for the young susceptible pullets housed with them (Dorsey & Harshfield, 1959). Clearly, in a similar manner, free flying birds that are chronically infected are a major risk (Glisson *et al.*, 2003). Transmission of the organism through the egg seldom, if ever, occurs (Glisson *et al.*, 2003).

Most species of farm animals may be carriers of *P. multocida* (Blackall, 2003). However, it is only the isolates of *P. multocida* from pigs and possibly cats that have been shown to be consistently pathogenic for fowl (Glisson *et al.*, 2003). It has been shown that healthy pigs that were carrying *P. multocida* were capable of infecting fowl held in the same enclosure (Glisson *et al.*, 2003).

Sparrows, pigeons and rats can become infected when exposed to chickens with fowl cholera and that they can then, in turn, infect susceptible chickens with fowl cholera (Serdyuk & Tsimokh, 1970). It is possible to artificially infect turkeys with fowl cholera by feeding them flies that had previously fed on *P. multocida*-infected blood (Skidmore, 1932). However, it appears that flies may not play a significant role in field situations. This was shown by Van Es and Olney (1940) who kept two pens of chickens with fowl cholera adjacent to pens of susceptible chickens at the height of the fly season. Despite the fact that fowl cholera was maintained in the two infected pens and the adjacent pens were separated by only chicken wire, the disease did not spread to the susceptible chickens (Van Es & Olney, 1940).

Contaminated crates, feed bags or any equipment used previously with poultry may serve as a means of introducing fowl cholera into a flock (Glisson *et al.*, 2003). When chickens die of fowl cholera, the carcass is heavily contaminated with live *P. multocida* and can serve as an infection source as fowl tend to consume such carcasses (Glisson *et al.*, 2003). The carcass of a chicken that died of fowl cholera has been shown to contain viable *P. multocida* for at least two months at 5-10^{0}C (Hendrickson & Hilbert, 1932).

Within a flock, the dissemination of fowl cholera is primarily due to excretions from the mouth, nose and conjunctiva of infected birds. These excretions contaminate the environment – particularly feed and water. Turkeys drinking from the same water trough as turkeys experimentally infected with *P. multocida* developed fowl cholera (Pabs-Garnon & Soltys, 1971).

Prevention and Control

Fowl cholera can be prevented by eliminating all reservoirs of infection within a property and then preventing any re-entry of the organism into the property. Implementation of standard good management practices, an effective sanitation regime and a good biosecurity program will mean that fowl cholera can be prevented (Blackall, 2003)

It is important to understand that fowl cholera is not transmitted via the egg. Hence, birds arriving from the hatchery are free of the disease. The producer has the task of ensuring that these clean and susceptible birds are not exposed to *P. multocida*.

The primary source of infection is usually sick birds or birds that have recovered BUT are still carrying the disease (Blackall, 2003). Only young birds should be introduced as new stock and they should be raised in a clean environment completely isolated from other birds. Older birds should never be mixed with younger birds. Different species of birds should never be raised on the same premises. Farm animals (particularly pigs, dogs and cats) must be excluded from the poultry area. Waterers should be self-cleaning or must be cleaned as regularly as possible. Feeders should be covered to prevent contamination. Wild birds must be prevented from associating with the flock. If an outbreak of fowl cholera occurs, the flock should be quarantined and disposed of as soon as economically possible. All housing and equipment associated with the infected flock must be cleaned and disinfected before repopulation occurs.

Vaccines can be used BUT they should not be substituted for a good management, good sanitation and good biosecurity. *P. multocida* exists in 16 different types – called serovars (Glisson *et al.*, 2003). Serovars 1, 3 and 4 are the most common serovars associated with fowl cholera outbreaks (Glisson *et al.*, 2003). Fowl cholera vaccines based on killed cells of *P. multocida* – which are currently the only vaccine type available in Australia – provide protection only against those serovars present in the vaccine (Glisson *et al.*, 2003).

Molecular Typing of *P. multocida*

Within the context of this project, typing is based on the theory that isolates of *P. multocida* that have a common source will share properties that allow these related isolates to be differentiated from other

non-related *P. multocida* isolates. A clear implication underlying this hypothesis is that the species *P. multocida* consists of genetically divergent lineages. There is considerable evidence that *P. multocida* is indeed genetically diverse e.g. on the basis of DNA:DNA hybridization and phenotypic properties three subspecies, *gallicida*, *multocida* and *septica*, have been recognised (Mutters *et al.*, 1985), NAD-dependent isolates of *P. multocida* have been recognised (Mutters *et al.*, 1985) and 16 different biochemical types, termed biovars, have been recognised within the species (Blackall *et al.*, 1997; Fegan *et al.*, 1995). Hence, there is potential for typing systems to achieve the goal of both grouping related isolates and separating unrelated isolates.

The traditional typing method has been serotyping. However, the discrimination power of serotyping is very low – i.e. many isolates from diverse origins share the same serovar. The use of DNA-based typing methods has been a major advance. These DNA typing methods, many of which have been developed and applied within the Microbiology Research Group using RIRDC funding, include restriction endonuclease analysis (REA), ribotyping, pulsed field gel electrophoresis (PFGE) and PCR methods such as ERIC-PCR and RAPD-PCR. A detailed review of these methods – including the advantages and disadvantages - has been published (Blackall & Miflin, 2000). While these methods have proven useful, they all share a major limitation – comparisons across laboratories and even across time are difficult to impossible to perform (Blackall & Miflin, 2000). This is a major limitation that leaves laboratories unable to share information – forcing repeated examinations or testing.

Multi-locus Enzyme Electrophoresis

Multi-locus enzyme electrophoresis (MLEE) is a typing method that detects variations in the amino acid sequence of enzymes associated with essential house-keeping activities of cells (Selander *et al.*, 1986). As such, MLEE is not a DNA-based method – it detects phenotypic variations rather than genotypic variations. However, MLEE has proven to be a very powerful typing tool. Studies performed at the Microbiology Research Group have demonstrated that the use of MLEE allowed the recognition of a population structure within avian *P. multocida* (Blackall *et al.*, 1998). The MLEE study has provided an insight into how "related" Australian avian *P. multocida* isolates are. This structure has been subsequently exploited to allow the rational selection of strains of known diversity for the evaluation of a new generation PCR-based diagnostic test (also funded by RIRDC) as well as strains for evaluation as virulent challenge organisms to evaluate new vaccines. The MLEE study has also shown that Australian isolates are as diverse as the 19 reference strains that were also studied – suggesting that Australian isolates of *P. multocida* are unlikely to be markedly different from those found in other countries (Blackall *et al.*, 1998). Work performed at the Microbiology Research Group has also shown that MLEE is as good as REA, ribotyping, PFGE and REP-PCR when performing molecular epidemiological studies on fowl cholera outbreaks (Blackall & Miflin, 2000).

However, as useful as this MLEE study has proven, MLEE suffers the same problem as the DNA based typing methods – comparisons across laboratories and across time are difficult if not impossible to perform. Hence, the MLEE study is now frozen in time – new, recent isolates cannot be added to the study.

Multi-locus Sequence Typing

The importance of *P. multocida* as a pathogen of birds and many other species is such that the complete chromosome of this pathogen was recently sequenced (May *et al.*, 2001). The completion of this task provides a unique opportunity to exploit this sequence knowledge. Multi-locus sequence typing (MLST) is a new generation typing method that takes MLEE to the level of a genotypic method (Spratt, 1999). Even more importantly, because MLST uses sequence knowledge, the typing achieved by MLST is totally portable – making comparisons across laboratories and across time easy and routine in nature (Enright & Spratt, 1998).

In basic terms, MLST is based on determining the DNA sequence changes that result in the amino acid changes detected by MLEE (Spratt, 1999). Essentially, rather than running gels to look for mobility variation in enzymes (as occurs with MLEE), MLST utilises DNA sequencing. The full power of MLST can be exploited by placing the data-base of MLST types onto a publicly accessible Web site (Enright & Spratt, 1998). This last feature means that once an MLST typing scheme has been established any laboratory can compare their isolate with any previously typed isolate by simply comparing the DNA sequences. All typed isolates stored in the central Web data-bank are accessible to any-one with Web access. The rapid progress in DNA sequencing technology means that this form of DNA-based typing is now feasible and indeed already functioning for major human pathogens such as *Neisseria meningitidis* (Maiden *et al.*, 1998) and *Campylobacter jejuni* (Dingle *et al.*, 2002). Indeed, it has now been claimed that MLST is the "gold standard" for typing bacterial pathogens (Maiden *et al.*, 1998).

Hence, there is a currently a unique opportunity available to establish MLST typing of *P. multocida*. The necessary genetic information to design the sequencing primers is now available. Due to the prior studies performed by the Microbiology Research Group, there is an existing culture collection of avian *P. multocida* that have been examined by MLEE. This existing MLEE structure will allow a rational selection of both isolates to be examined and which genes should be targeted for sequencing.

Overall, it is now possible to establish a rapid, specific and sensitive DNA sequence-based typing method that will support the development of informed, effective, sustainable prevention and control programs for fowl cholera. This project will also allow the establishment of a cutting edge typing technology that represents the future of typing. This is a unique opportunity to combine the power of recent advances in DNA technology with the power of interactive Web technology.

Relevance to the chicken meat and egg industries

The Australian chicken meat industry currently produces approximately 715,000 tonnes of chicken meat annually with a retail value in excess of $3.6 billion (Anonymous, 2005a). An annual growth in production of between three and four per cent has been experienced by the industry in recent times and similar levels of growth in production volume are expected to continue for at least the next two to three years (Anonymous, 2005a). A current strategy of the Australian chicken meat industry is to "continue to develop improved disease prevention, management and diagnostic techniques for economically important existing and emerging endemic diseases and for emergency diseases" (Anonymous, 2005a).

The farm gate value of the Australian egg industry is around $330 million per year (Anonymous, 2005b). A key long term priority of the Australian egg industry is to develop strategies and technologies to rapidly detect and control infectious and non-infectious diseases of concern to the egg industry (Anonymous, 2005c).

This project is a good example of the development of technologies to support improved disease prevention programs and thus fits well into the research priorities of both the chicken meat and the egg industries. An ability to rapidly and confidently type isolates of *P. multocida* combined with the ability to compare that type with all other typed isolates via a Web-based data-base is a powerful tool in the development of sustainable and cost effective prevention and control programs.

Objectives

- Establishment of Multi-locus Sequence Typing (MLST) of *Pasteurella multocida*
- Rapid, accurate typing of *P. multocida* isolates that will allow any isolate to be directly compared with any previous isolate already typed and allow an understanding of the epidemiology of fowl cholera outbreaks
- Improved prevention and control programs for fowl cholera

Chapter 1: Materials and Methods

Introduction

This Chapter provides full details of the various materials and methods used in this study.

Bacterial Strains

A total of 66 organisms previously identified as *P. multocida* were used in this study. The cultures were obtained from the culture collection of the Microbiology Research Group at the Animal Research Institute (Yeerongpilly, QLD). Table 1 displays the available information of *P. multocida* isolates. The somatic serovar, biovar and electropheretic type (ET) details are as provided by Blackall *et al.* (1998). Three of the isolates were the formal taxonomic reference strains for *P. multocida* subspecies *multocida* (PM55 NCTC 10322), *P. multocida* subspecies *gallicida* (PM57 NCTC 10204) and *P. multocida* subspecies *septica* (PM59 CIP A125). All the remaining 63 isolates were from Australian poultry.

Media

Blood agar (Blood Agar Base - BBL/Becton Dickinson, 4311037 containing 5% Sheep Blood) was used for the routine growth of *P. multocida* isolates.

Revival of strains

Most of the *P. multocida* strains were revived from stocks held frozen at -70° C. Ice crystals were scraped from the surface of the frozen stock and inoculated onto Sheep Blood Agar (SBA) and also into 5 ml of Nutrient broth (NO.2 Oxoid) (NB).

Some of the strains were revived from freeze dried ampoules. The ampoules were disinfected with 70% ethanol before opening. The freeze-dried tube was opened by first weakening the glass at the mid-level of the air filter cotton wool plug by scratching with a glass-cutter. This weakened area was then cracked open by applying hot molten glass to the scratch line. The cotton wool was then soaked in a small volume of NB. The suspension was then used to inoculate SBA and a 5 ml NB.

Both the inoculated SBA and NB were incubated overnight at 37^{O}C. If the SBA showed typical growth of *P. multocida*, the NB was discarded. If the SBA showed no typical *P. multocida* growth, then the NB was inoculated onto an SBA plate and the plate incubated at 37^{O}C.

Confirmatory Phenotypic Identification

The revived cultures were subcultured onto SBA and incubated overnight at 37^{O}C. These plates were carefully examined visually for contamination and the following phenotypic tests were done to confirm the identification of the culture. The catalase test was performed as previously described (Barrow & Feltham, 2000). The oxidase test was performed using the Bactidrop Oxidase reagent (Remel, Cat # 21540) as directed by the manufacturer. The Gram stain was determined by either performance of the Gram stain using the Jensen's modification (Cruickshank *et al.*, 1975) or by testing for increased viscosity when the culture was emulsified in 3% KOH (Manafi & Kneifel, 1990). The indole reaction was performed by placing a drop of Kovác's indole reagent (Merck, Cat # 1.09293.0100) onto a piece of filter paper. The culture was smeared onto the area wetted by the indole reagent. A pink colouration within 20 seconds was recorded as a positive reaction.

Genomic DNA Extraction

P. multocida genomic DNA was extracted using the DNeasy™ Tissue Kit (QIAGEN Cat # 69506). The protocol for animal tissues as described in the kit instructions was used for all extractions. Prior to the use of the kit, a cell suspension was prepared for each isolate to provide a clean, acceptable cell source, of the correct concentration, for extraction of DNA.

A 1 µl loopful of pure *P. multocida* colonies from a one-day-old culture on SBA was picked and washed into 500 µl of sterile phosphate buffered saline (PBS) by stirring with the loop and vortexing. Next, the solution was centrifuged for 10 minutes at 5,000 *g*. Then the supernatant was discarded and the pellet resuspended in 500 µl of PBS by vortexing.

The above cell suspension was transferred in small volumes into 4 ml of PBS until the density matched that of a MacFarland No 1 tube (equivalent to 3×10^8 cells/ml). A 1 ml aliquot of this suspension was transferred to a microfuge tube and centrifuged for 10 minutes at 5,000 x g. The supernatant was discarded and the pellet was resuspended in the buffer provided in the kit. The extraction of DNA was then carried out using the DNeasy Protocol for Animal Tissues. The 200 µl elute was aliquoted into 2 X 100 µl volumes and kept at –70° C. Later, one 100 µl tube was taken out and aliquoted into 5 X 20 µl volumes and kept at –20° C for day to day use.

The extracted DNA was visualized for quality and quantity by electrophoresis in a 2% DNA grade agarose gel in 1 × TAE (Tris Glacial Acetate EDTA) buffer, stained with ethidium bromide (10 mg/ml) and photographed using ultra-violet light. A low DNA mass ladder (Invitrogen 10068-013) was included in the gel to estimate the quantity of the extracted DNA.

Some of the DNA sample concentrations were quantified using Fluorescent DNA Quantitation kit (BioRad) as per the manufacturer's instructions to determine the quality of the DNA extractions.

Genotypic Identification using *P. multocida* species specific PCR

The extracted DNA was used in a species-specific PCR assay for *P. multocida*. The PCR of Miflin and Blackall (2001) was performed as previously described. A positive control (*P. multocida* subspecies *multocida* - PM55 - NCTC 10322) and a negative control (sterile water) were included in every set of reactions.

A 10 µl aliquot of the PCR reaction was mixed with 2 µl loading buffer and then run in 1% DNA-grade agarose gel containing ethidium bromide (1µl/µg) in 1%TAE buffer at 80 V for 30 minutes and visualised under UV light. Ready-load 100 bp DNA ladder (Invitrogen Cat # 10380-012) was used in all gels to determine fragment size.

A positive result was a band of approximately 1430 bp (Miflin & Blackall, 2001).

Selection Of House Keeping Genes

The selection of house keeping genes was based on the genetic diversity of the relevant enzymes as shown in the prior MLEE study (Blackall *et al.*, 1998). The *P. multocida* genome sequence database, located at National Centre for Biotechnology Information (NCBI) website [<http://www.ncbi.nlm.nih.gov/genomes/framik.cgi?db=genome&gi=170>] was searched for annotated nucleotide sequence data. The five genes selected from the pre-existing MLEE study that could be confidently identified on the annotated genome were *est, mdh, pgi*, *pmi* and *zwf*.

Existing MLST schemes for *N. meningitidis* (Maiden *et al.*, 1998) and *Streptococcus pneumoniae* (Enright & Spratt, 1998) were also consulted to aid in selection of the final two genes. The two genes selected on this basis were *adk* and *gdh*.

Details of the selected genes are presented in Table 2.

Designing of Primers

All primer sets used in this study were designed using the Primer 3 primer designing tool on the internet, located at http://biotools.umassmed.edu/bioapps/primer3_www.cgi. All primers were designed against the nucleotide sequence of respective gene in the PM 70 strain, the strain that has been fully sequenced and deposited at the NCBI.

The nucleotide sequences of the relevant genes were entered into the Primer 3 designing tool. The following design parameters were used:- length 18-22 bp, Tm 58-62°C, two GC clamps, no ambiguous bases, the 3' end should finish with a G/C (Buck *et al.*, 1999) and the 3' end should not have a hairpin (Buck *et al.*, 1999).

The details of the eight primer sets used in this work are presented in Table 3. Two primer sets were used for *zwf*.

Both forward and reverse primers of each candidate primer pair were compared with the general nucleotide database at the NCBI website using the program BLAST. As well, alignments against the *P. multocida* genome were performed to ensure that only one amplicon would be produced for each primer set.

Evaluation of primers and optimisation of PCR conditions for the MLST-Enzyme PCRs

Initially, six isolates of *P. multocida* were chosen randomly to determine that each primer set could produce a single amplicon. PCR reactions over a range of annealing temperatures were performed in order to determine the optimal Tm for each primer set. The 50 µl PCR reaction mixture contained 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris HCl (pH 8.3), 200 µM of each dNTP (dATP, dCTP, dGTP, dTTP), 0.5 µM of each primer and 1.25 units Taq DNA polymerase (Boehringer Mannheim, Mannheim, Germany). Two µl of genomic DNA (see above) was used as template.

For all PCRs except the PCR for *gdh*, the following reaction conditions were used :- 30 seconds of denaturation at 94° C, 30 seconds of annealing at 48° C and 1 minute of extension at 72° C for 30 cycles. A last cycle of elongation for 5 minutes at 72° C was used. For the *gdh* PCR, the only change was that the annealing temperature was 58° C. For all PCR reactions, an initial denaturation of 5 minutes at 94° C was performed.

A 4 µl aliquot of the PCR product was mixed with 1 µl loading buffer and then run in 2% DNA-grade agarose gel containing ethidium bromide (1 µl/µg) in 1%TAE buffer at 80 V for about 40 minutes and visualised under UV light. Ready-load 100 bp DNA ladder (Invitrogen 10380-012) was used in all gels to determine fragment size.

A positive result was a single band of expected size product of respective primer set predicted by Primer 3 design tool.

PCR-product purification

Two successful products of the same reaction were combined to give a 100 µl total volume. The QIAquick PCR purification kit (QIAGEN Cat.No. 28104) was used to purify the product. The procedure was carried out as in the protocol except that 30 µl of elution buffer was used to elute the DNA instead of 50 µl (resulting in a more concentrated DNA preparation).

Initially, some samples were subjected to a 1% agarose gel along with low DNA mass ladder (Invitrogen, Cat.No.10068-013) to determine the concentration of the purified product.

Sequencing PCR reaction

The sequencing PCR reaction was carried out using the PRISM Ready Reaction Dyedeoxy™ Terminator Sequencing kit (Applied Biosystems, Australia). BigDye Terminator (BDT) version 3.1 and the BDT buffer were supplied in the kit. The PCR was performed as described in the SequencingKit.

For each PCR amplified gene fragment two sequencing reactions were carried out separately, one with the forward primer and one with the reverse primer.

The sequencing products were ethanol precipitated to separate the amplified DNA from the leftover reaction mix. The protocol for precipitation in microfuge tubes (Applied BioSciences hand book) was followed. Non-denatured 95% DNA grade ethanol was stored in the freezer and used ice-cold. As required, 70% ethanol was made fresh. The precipitated DNA was air dried in a Class II Biohazard Hood.

The microfuge tube containing the dried DNA precipitate was wrapped in aluminium foil (for protection against the light) and sent to Griffith University DNA Sequencing Facility (GUDSF) for sequencing.

Sequence Compilation

The raw sequence chromatograms were obtained from GUDSF and visually screened with editing software named EditView (v.0.1 ABI PRISM) for the quality of sequences. Initially sequences were examined in NCBI with BLAST using basic nucleotide comparison (blastn) option for the confirmation of identity until the alignments for each gene was built up in the Sequence Alignment software (SeqED, v 1.0.3).

The sequences were imported into SeqED. The forward and reverse complimented sequences were aligned and carefully cross-checked for complementarity. The ends of the sequences were then trimmed as MLST analysis only requires gene fragments of 450 bp length. For each housekeeping gene analysed, a multiple alignment was manually created in SeqEd from all of the isolates sequenced. The edited sequences were saved into a text file for analysis. The sequence files were later converted into different formats such as FASTA for analysis using the START suite of programs (Sequence Type Analysis and Recombination Tests, available at the MLST website http:/pubmlst.org/software/analyis/).

Assignment of Allelic Profiles

For each gene fragment, different sequences were assigned as distinct alleles. This resulted in a 7-digit allelic profile for each isolate. The allelic profiles obtained were generated by the software package BioNumerics. Each unique allelic profile was manually assigned as a sequence type (ST) with a random number. Isolates with the same allelic profile were assigned as the same ST.

The START suite of programs were downloaded from the MLST website. The allelic profiles and the STs were then used in the MLST data analysis within the START suite. The instructions for START analysis were obtained from the MLST website (http:/pubmlst.org/software/analyis/). The construction of a dendogram showing the unweighted pair group method with arithmetric averages (UPMA) was also performed with the START program.

Pasteurella multocida MLST database

The MLST data generated in this work (allelic profiles, ST's, enzyme profiles and isolate profiles) were submitted to the MLST website as an Access data base. The data then used to construct the *P. multocida* MLST website, which was kindly done by Dr. David Aanensen, the curator of the MLST website.

Table 1. Isolates of *P. multocida* used in this study. A total of 63 of the isolates were obtained from Australian poultry. Three strains (PM 55, PM57 and PM59) are formal reference strains for the three subspecies within *P. multocida*. All the Australian isolates were associated with outbreaks of fowl cholera.

Study Code	Source	Year of Isolation	Somatic Serovar[A]	Biovar[B]	Ribotype & Cluster[C]	ET & Cluster (MLEE)[C]
PM1	Turkey	1993	3,4	2	6/R1	42/B
PM2	Turkey	1993	4	3	7/R4	38/A
PM3	Turkey	1993	4,10,15	3	7/R4	38/A
PM4	Turkey	1993	3	3	7/R4	38/A
PM5	Turkey	1993	NT	3	7/R4	38/A
PM6	Turkey	1993	NT	3	7/R4	38/A
PM7	Turkey	1993	NT	3	7/R4	38/A
PM8	Turkey	1993	10	3	7/R4	38/A
PM9	Turkey	1993	NT	3	7/R4	38/A
PM10	Turkey	1993	4	3	7/R4	38/A
PM11	Turkey	1993	NT	3	7/R4	38/A
PM12	Turkey	1993	3,4	2	6/R1	43/B
PM13	Turkey	1993	3	2	6/R1	43/B
PM14	Turkey	1993	3	2	6/R1	43/B
PM15	Turkey	1993	11,12	3	7/R4	38/A
PM16	Turkey	1993	11,12,15	3	7/R4	38/A
PM17	Turkey	1993	3	2	7/R4	45/B
PM18	Chicken	1986	3	3	10/R2	22/A
PM19	Turkey	1986	3	3	5/R3	31/A
PM27	Chicken	1986	3	3	2/R5	19/A
PM35	Turkey	1986	3	3	5/R3	7/A
PM37	Chicken	1988	3	1	12/R7	6/A
PM40	Chicken	1988	3	3	5/R3	7/A
PM45	Chicken	1986	3,4	3	5/R3	11/A
PM46	Chicken	1992	6	1	7/R4	3/A
PM48	Chicken	1983	3,4	3	2/R5	18/A
PM49	Turkey	1984	1,15	1	14/R6	9/A
PM51	Chicken	1984	4,12	3	11/R5	13/A
PM52	Chicken	1983	3,4,12	3	2/R5	20/A
PM55 (NCTC 10322)	Porcine	1962 Canada	NK	11	17/R6	R12/A
PM57 (NCTC 10204)	Bovine	1960 UK	NK	2	13/R7	R13/A
PM59 (CIP A125)	Human (Cat bite)	1952 France	NK	7	6/R1	R15/B
PM63	Chicken	1976	3	3	2/R5	1/A
PM64	Chicken	1979	3	5	9/R8	16/A
PM65	Chicken	1979	4	5	9/R8	16/A
PM67	Turkey	1969	3,12	4	13/R7	14/A
PM69	Chicken	1973	NT	3	5/R3	32/A
PM71	Chicken	1979	4	6	20/R5	5/A
PM72	Chicken	1977	3,14	6	4/R1	51/B
PM73	Turkey		4	3	2/R5	2/A
PM75	Duck	1979	3,4,12,14	3	5/R3	29/A

Study Code	Source	Year of Isolation	Somatic Serovar[A]	Biovar[B]	Ribotype & Cluster[C]	ET & Cluster (MLEE)[C]
PM76	Chicken	1979	NT	5	9/R8	15/A
PM77	Chicken	1980	3	3	10/R2	21/A
PM78	Chicken	1981	3	3	5/R3	29
PM79	Chicken		1	8	15/R2	39/A
PM80	Chicken		3,4,12	3	5/R3	26/A
PM81	Chicken		NT	6	4/R1	52/B
PM83	Chicken		NT	1	12/R7	4/A
PM84	Chicken		NT	1	12/R7	37/A
PM85	Turkey	1988	3	3	5/R3	7/A
PM86	Turkey	1988	3	3	5/R3	7/A
PM87	Chicken	1986	3	3	5/R3	11/A
PM88	Chicken	1986	NT	1	3/R6	10/A
PM91	Chicken	1986	3	2	6/R1	44/B
PM95	Chicken	1991	3	3	5/R3	28/A
PM96	Chicken	1991	3	4	13/R7	17/A
PM97	Turkey	1991	NT	3	11/R5	34/A
PM120	Turkey		12	3	11/R5	12/A
PM131	Chicken		1	2	15/R2	55/B
PM132	Turkey	1992	4	3	2/R5	33/A
PM133	Turkey	1992	4	3	1/R1	27/A
PM135	Turkey	1992	13	10	4/R1	47/B
PM136	Turkey	1992	3	3	5/R3	25/A
PM137	Turkey	1992	4,7	3	1/R1	36/A
PM138	Chicken	1994	1,15	2	4/R1	54/B
PM140	Chicken		13,14,15	7	4/R1	48/B

[A] As reported by Blackall *et al.* (1998). NT = Non-typable, NK = Not known.
[B] As reported by Blackall *et al.* (1998) using the biovars defined by Fegan *et al.* (1995)
[C] As established by Blackall *et al.* (1998)

Table 2. Details of the house keeping genes utilized in this study

House keeping Gene	Abbreviation	NCBI No.[A]	Diversity MLEE[B]	Position[C]	Size (bp)	Known Function
Adenylate kinase	*adk*	PM0284	0.227	323720-324364	645	Nucleotide biosynthesis
Esterase	*est*	PM0076	0.871	102920-104959	2040	Hydrolase
Mannose-6-Phosphate Isomerase	*pmi*	PM0829	0.833	978551-979753	1205	Isomerase
Glucose-6-Phosphate Dehydrogenase	*zwf*	PM1549	0.416	1751723-1753213	1493	Energy metabolism: the pentose phosphate pathway
Malate Dehydrogenase	*mdh*	PM0002	0.416	983-3259	759	Energy metabolism: the TCA cycle
Glutamate dehydrogenase	*gdh*	PM0043	0.200	45666-47015	1350	Glycolysis
Phospho Glucose Isomerase	*pgi*	PM0416	0.656	488453-490102	1652	Energy metabolism: glycolysis

[A] Number as assigned at the NCBI Website
[B] Diversity of this gene as calculated in the MLEE study performed by Blackall *et al.* (1998)
[C] Position of the gene within the genome as recorded at the NCBI Website and based on the work of May *et al.* (2001)

Table 3. Primer sets designed for the amplification and sequencing of seven *P. multocida* housekeeping genes.

Gene	Primer Sequence (5' to 3')		Amplicon Size (bp)
	Forward	Reverse	
adk	TTTTTCGTCCCGTCTAAGC	GGGGAAAGGGACACAAGC	570
est	TCTGGCAAAAGATGTTGTCG	CCAAATTCTTGGTTGGTTGG	641
pmi	TGCCTTGAGACAGGGTAAGC	GCCTTAACAAGTCCCATTCG	739
zwf	AATCGGTCGTTTGACTGAGC	TGCTTCACCTTCAACTGTGC	808
	TGTTAGGTGTGGCAAGAACG	TTGCAACAAATGGTTTTGGA	614
mdh	ATTTCGGGATCAGGGTTAGC	GGAAAACCGGTAATGGAAGG	620
gdh	ATCGACTTCTTCCGCAGACC	GCGGGTGATATTGGTGTAGG	702
pgi	ACCACGCTATTTTTGGTTGC	ATGGCACAACCTCTTTCACC	784

Chapter 2: Results

Introduction

This Chapter presents the detailed results obtained during this project.

Identification tests

All 66 strains gave the expected pattern of results for *P. multocida*. All the strains were positive in the catalase, indole and oxidase tests. The isolates were all Gram-negative cocco-bacilli (by either the traditional Gram stain or the KOH test).

All the strains gave a single specific band of the expected size (1,430 bp) in the *P. multocida* PCR.

Gene selection

The original plan was that all seven genes would be selected on the basis of the known diversity established in the MLEE study of Blackall *et al.* (1998). However, great difficulty was encountered in identifying the relevant genes at the NCBI website. This difficulty arose from the fact that the gene naming convention adopted in typical MLEE studies such as Blackall *et al.* (1998) is not the same convention as adopted by those researchers annotating genome sequences.

Hence, only five genes selected as being of high diversity in the MLEE study (Blackall *et al.*, 1998) could be identified on the annotated genome of *P. multocida* at the NCBI website - *est, mdh, pgi, pmi* and *zwf*. The remaining two genes, *adk* and *gdh*, were selected as these genes have been commonly used in other MLST schemes (Enright & Spratt, 1998; Maiden *et al.*, 1998).

MLST-Primers

Eight sets of primers were designed to accomplish the task of sequencing all seven gene fragments of housekeeping genes from the 66 strain. Each gene was amplified and sequenced with a single set forward and reverse primers with the exception of the *zwf* gene. Two sets of primer pairs were necessary for this gene due to a possible mutation at forward primer site for ten strains. The second set of primer pair was designed within the first primer sites and thus produced a smaller amplicon.

Primer Optimization

A gradient PCR was done to find the ideal conditions to amplify the *est* gene product. The annealing temperatures tested ranged from 48.1 ^{0}C to 60.2^{0}C while the $MgCl_2$ concentrations ranged from 1.5 to 2.5 mM. A product was amplified in all the tested conditions, thus the conditions in which the strongest amplification occurred (i.e. with the brightest band intensity) were chosen as the final PCR conditions for the *est* PCR. The rest of the six gene PCRs were initially run using these conditions. All other set of primers gave good results in the same condition as the *est* gene PCR except the *gdh* gene PCR. For the *gdh* PCR, the gradient experiments revealed that a higher annealing temperature was necessary.

For all 66 strains, PCR products of the expected size were obtained for six genes (*est, mdh, pgi, pmi, adk* and *gdh*) using the single primer set for each respective gene shown in Table 3 (in Chapter 2). However, for *zwf* gene, the initial primer set (ZWF-F1 and ZWR-R1) failed to give a product for 10 strains. As noted above, a second set of primers were designed and these primers resulted in a product of the expected size for these 10 strains.

Sequencing of housekeeping genes

The seven fragments were sequenced from all 66 *Pasteurella* strains. Gene sequence results were only accepted if the chromatogram showed sharp clean peaks with a low noise level. Repeat sequencing was performed as necessary. Alignment of all sequences obtained with each primer set for all 66 strains was performed. For the purpose of the MLST analysis, all sequences were restricted to the first 450 bases.

Allelic variation

The sequence diversity within the *Pasteurella* genes was sufficient to distinguish 9-21 alleles among the seven gene fragments. In many cases, alleles differed from each other at multiple nucleotide sites.

The distribution of the 66 strains across the various alleles for all seven genes is shown in Table 4. The gene with the most alleles was *est* (21 alleles) while the gene with the least alleles was *mdh* (nine alleles). Further details of the diversity present in each gene locus is shown in Table 5. Nucleotide sequence diversity within the gene loci varied from 1% (*adk)* to 8.5% (*est*). The polymorphic sites within the *zwf* locus are shown in Figure 1.

Assignment of allele and sequence types

Each distinct sequence (allele sequence) at a particular locus was assigned a unique arbitary number (an allele type). The numbers of difference alleles resolved at each locus varied from 9 (*mdh*) to 21(*est*).

After sequencing and assignment of allele types to all seven loci, each strain was then designated by a combination of seven numbers, called an allelic profile, in the order of *adk, est, pmi, zwf, mdh, gdh* and *pgi* which represented a sequence type (ST) for that particular strain. Subsequent isolates with an identical allelic profile were assigned to the same ST and considered to be isogenic as they were indistinguishable at all seven loci. A total of 39 STs were recognised within the 66 *P. multocida* strains in this study (see Table 6).

The relationship between each ST was displayed in a dendogram (Figure 2). The linkage distance in the dendrogram is expressed as the mean allelic diversity over all loci. If the value approaches one, it indicates a high degree of dissimilarity or heterogeneity within the population, whereas values closer to zero indicate a high degree of similarity or homogeneity (Yakubu *et al.*, 1999).

The dendogram has two major Clades A and B, separated at a linkage distance of 1, indicating a high discrimination between Clades A and B. A summary of the properties within each of these two Clades is provided in Table 7.

Correlation between serovars, biovars, geographical origin and ST designation

The following comparisons are based on the serovars and biovars as reported by Blackall *et al.* (1998).

No isolates of biovar 3, which was by far the most common biovar (39/66 strains), were located in MLST Clade B. Indeed, the biovars appeared to be grouped in specific clusters, with biovars 1, 3, 4, 5, 8 and 11 in MLST Clade A and biovars 6, 7 and 10 in MLST Clade B. Only biovar 2 occurred in both Clades with seven strains being allocated to Clade B and two strains being allocated to Clade A. Within the eight STs that consisted of more than one isolate (STs 1, 2, 7, 8, 19, 22, 34 and 36), all members were always allocated to a single biovar with the exception of ST 2. In ST 2, one strain (PM 17) is biovar 2 while the other 11 members of this ST are biovar 3.

In terms of the recognised subspecies of *P. multocida*, the MLST analysis placed all three strains belonging to the subspecies *septica* (biovars 7 and 10) in Clade B. Five of the seven strains belonging to the subspecies *multocida* that produce acid from sorbitol, trehalose and xylose (biovar 2) were also placed in Clade B.

In contrast, there appears to be no obvious division of serovars between MLST Clades A and B. Serovar 3 was the most common serovar overall and was the most common serovar in both MLST Clades A and B.

The three STs containing the taxonomic reference strains did not include any Australian field isolate. Furthermore, two of the STs containing the reference strains (ST 13 labelled as Clade Z in Figure 2; ST 14 labelled as Clade Y containing the reference strain for subspecies *multocida* and subspecies *gallicida* respectivey) were markedly separated from the Australian strains – not linking with any Australian strain till a distance of 0.7 or 0.85 (subspecies *multocida* and subspecies *gallicida* respectivey). Only the reference strain for the subspecies *septica* (ST 15 – labelled as Clade X in Figure 2) joined with an Australian isolate at a linkage distance of less than 0.5.

STs and ribotypes

The following comparisons are based on the ribotypes and ribotype clusters as reported by Blackall *et al.* (1998).

There was a strong correlation between the MLST and ribotyping results. Within the eight STs that consisted of more than one isolate (STs 1, 2, 7, 8, 19, 22, 34 and 36), all members were always allocated to a single ribotype. In addition, where different STs shared a ribotype, the dendogram indicated that these STs were closely related (typically differing in only one allele). As an example, STs 2 and 4 had the same ribotype (ribotype 7). Figure 2 indicates that these two STs join at a linkage distance of 0.15. Table 6 also indicates that STs 2 and 4 differ only in the *pgi* locus. There are other examples of this same occurrence i.e. STs that share a common ribotype are typically closed placed in the dendogram and differ in only one allele e.g. STs 6 and 8 and STs 16 and 17. A particularly strong example of shared ribotypes across very closely related STs occurs with STs 19, 29 and 30. All three STs share the same ribotype (ribotype 5 within ribotype cluster R3) and all are closely placed in Figure 2 (ST 19 is marked as Clade N).

At the level of the MLST Clades and ribotype clusters there was also good agreement. Ribotype Cluster R1 was almost a perfect correlation with MLST Clade B. All 12 strains in MLST Clade B are within the Ribotype R1 cluster. More specifically, all strains of ribotypes 6 (six strains) and 4 (six strains) are located within ribotype cluster R1 and are also the sole members of MLST Clade B. Ribotype 1 is within Ribotype cluster 1 but the two members of this ribotype examined in this study were assigned to MLST Cluster A. MLST Cluster B contained all the other ribotype clusters (R2, R3, R4, R5, R6, R7 and R8).

STs and Electrophoretic Types

The following comparisons are based on the electrophoretic types (ETs) and MLEE clusters as reported by Blackall *et al.* (1998).

There was very strong agreement at the clustering level between MLST and MLEE. Of the 13 strains in this study assigned to MLEE Cluster B, 12 were assigned to MLST Clade B. These 12 strains are the only members of MLST Clade B. The remaining 53 strains examined in this study were assigned to MLEE Cluster A and were all assigned in the current study to MLST Clade A. The only strain that was not matched between the MLST and MLEE studies was PM 17.

When comparing STs and ETs, there are some disagreements and some agreements between the systems. The MLST system recognised eight STs with more than one strain. Of these eight multi-strain STs, only one contained strains that were all also assigned to a single ET. This multi-strain ST with matching ET distribution is ST2. Within ST 2, there is one exceptional strain – PM17, a strain that was assigned to a very different ET to all other members of this ST. One other multi-strain ST, ST 1, contained strains that were assigned to two closely related ETs (ETs 42 and 43). The remaining six multi-strain STs contained strains that were typically from quite diverse ETs. An example is ST 8 (which is marked as Clade M in Figure 2). This ST consisted of four strains, two assigned to ET 7, one assigned to ET 11 and the last one assigned to ET 29. These three ETs came together at a genetic distance of 0.25 in the MLEE study (Blackall *et al.*, 1998).

Table 4. Distribution of the 66 strains of *P. multocida* amongst the various alleles of the seven gene loci used in this study.

Allele	**Number of *P. multocida* strains with the indicated allele for the following gene loci**						
	adk	***est***	***pmi***	***zwf***	***mdh***	***gdh***	***pgi***
1	5	5	5	8	9	10	5
2	19	13	27	14	13	15	12
3	1	2	3	2	1	20	1
4	5	14	4	7	11	6	3
5	1	8	4	13	25	4	13
6	1	3	1	2	3	1	5
7	1	1	5	1	2	1	3
8	1	1	1	3	1	5	10
9	1	4	3	3	1	1	1
10	2	1	3	1	-	1	1
11	11	2	1	1	-	2	3
12	6	1	2	3	-	-	2
13	2	2	3	2	-	-	2
14	1	1	1	1	-	-	1
15	3	1	2	1	-	-	1
16	4	1	1	1	-	-	2
17	2	1	-	1	-	-	1
18	-	2	-	2	-	-	-
19	-	1	-	-	-	-	-
20	-	1	-	-	-	-	-
21	-	1	-	-	-	-	-
Total alleles for each gene	17	21	16	18	9	11	17

Table 5. Analysis of the seven MLST loci in the 66 *P. multocida* strains examined in this study

Gene	No. of alleles	No. of polymorphic sites	Proportion of polymorphic sites (%)
adk	17	5	1
est	21	38	8.5
gdh	11	28	6
mdh	9	8	2
pgi	17	6	1.5
pmi	16	16	3.5
zwf	18	25	5.5

```
  1       10        20        30        40        50
  ATTTTGTAGCCACCTTTATTATCAGGCATTAAATACCGCGGATGCTGCCG
                                            A

 51       60        70        80        90       100
  ATTATGGCAAGTTAATTCCTCGTCTTGATGACTTACATGATAAATATCAA
                                  T

101      110       120       130       140       150
  ACCTGTGGTAACACGCTTTACTATTTATCCACGCCGCCAAGCCTTTATGG
                A                           T

151      160       170       180       190       200
  CGTGATTCCAGAATGCCTTGCGGCACATGGGTTAAATACTGAAGAGTTTG
  T              T     T  G  C  AC G     C     A

201      210       220       230       240       250
  GCTGGAAACGGTTAATTGTGGAAAAACCGTTTGGTTATGATATCCGCACG
   T     G                                         C

251      260       270       280       290       300
  GCAAAAGAACTCGATATTCAAATTCACCGTTTCTTTGATGAACACCAAAT
          T G  CG

301      310       320       330       340       350
  TTATCGTATTGACCACTATCTTGGTAAAGAAACGGTTCAAAATTTGCTCG
                                    A         C    T

351      360       370       380       390       400
  TGTTGCGTTTTTCTAATGGATGGTTTGAACCACTCTGGAACCGTAATTTC

401      410       420       430       440       450
  ATTGATTATATTGAAATCACGGGCGCAGAATCTATCGGTGTAGAAGAGCG
                A
```

Figure 1. Polymorphic sites within the *zwf* locus.

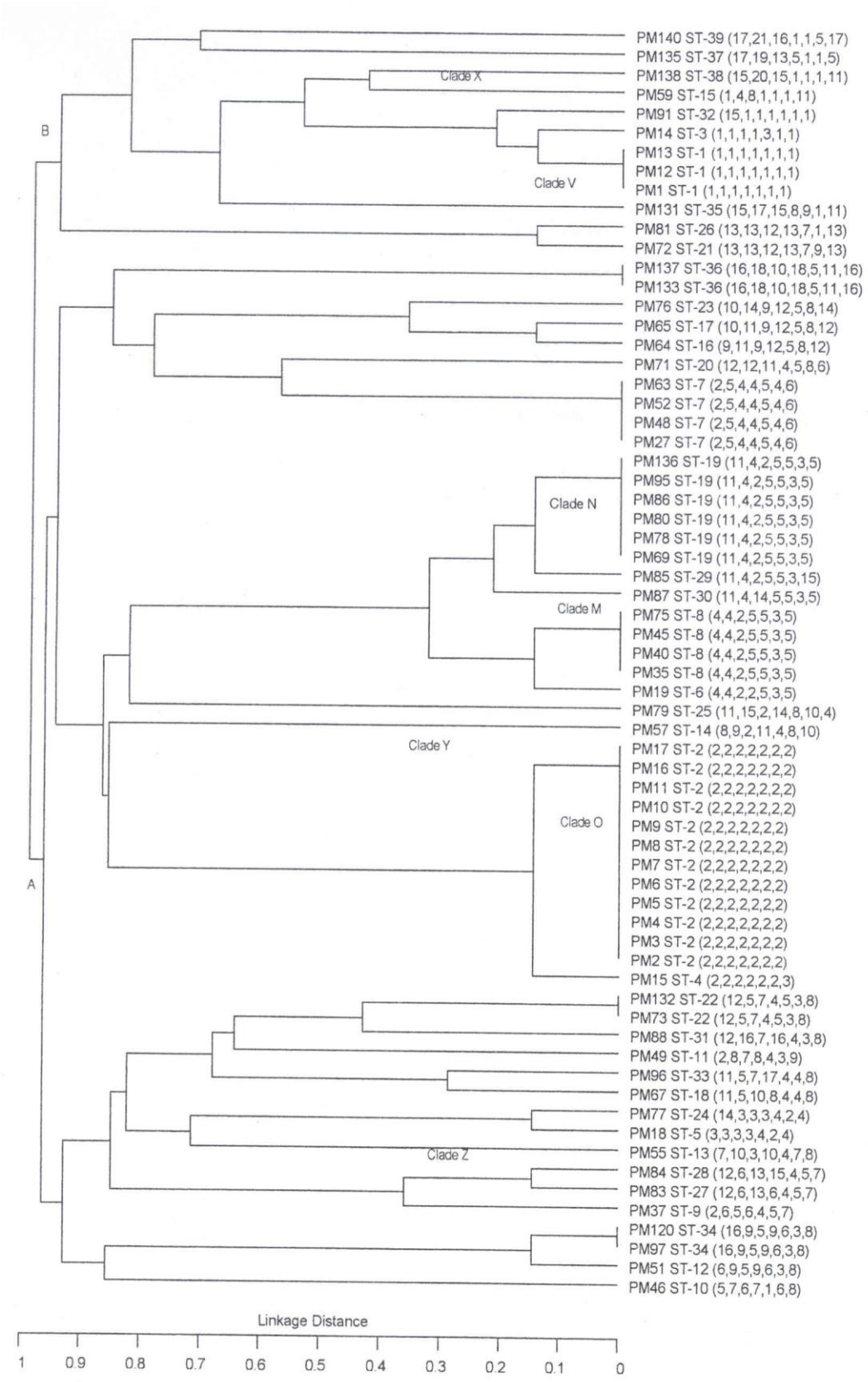

Figure 2. Dendogram of the 39 STs of *P. multocida* recognised in this study.

Table 6. MLST analysis of the isolates of *P. multocida* used in this study showing the known characteristics of the strains.

ST & Cluster	Strain	Source	Year of Isolation	Somatic Serovar[A]	Biovar[B]	Ribotype & Cluster[C]	ET & Cluster (MLEE)[C]	Allelic profile						
								adk	*est*	*pmi*	*zwf*	*mdh*	*gdh*	*pgi*
1/B	PM1	Turkey	1993	3,4	2	6/R1	42/B	1	1	1	1	1	1	1
	PM12	Turkey	1993	3,4	2	6/R1	43/B	1	1	1	1	1	1	1
	PM13	Turkey	1993	3	2	6/R1	43/B	1	1	1	1	1	1	1
2/A	PM2	Turkey	1993	4	3	7/R4	38/A	2	2	2	2	2	2	2
	PM3	Turkey	1993	4,10,15	3	7/R4	38/A	2	2	2	2	2	2	2
	PM4	Turkey	1993	3	3	7/R4	38/A	2	2	2	2	2	2	2
	PM5	Turkey	1993	NT	3	7/R4	38/A	2	2	2	2	2	2	2
	PM6	Turkey	1993	NT	3	7/R4	38/A	2	2	2	2	2	2	2
	PM7	Turkey	1993	NT	3	7/R4	38/A	2	2	2	2	2	2	2
	PM8	Turkey	1993	10	3	7/R4	38/A	2	2	2	2	2	2	2
	PM9	Turkey	1993	NT	3	7/R4	38/A	2	2	2	2	2	2	2
	PM10	Turkey	1993	4	3	7/R4	38/A	2	2	2	2	2	2	2
	PM11	Turkey	1993	NT	3	7/R4	38/A	2	2	2	2	2	2	2
	PM16	Turkey	1993	11,12,15	3	7/R4	38/A	2	2	2	2	2	2	2
	PM17	Turkey	1993	3	2	7/R4	45/B	2	2	2	2	2	2	2
3/B	PM14	Turkey	1993	3	2	6/R1	43/B	1	1	1	1	3	1	1
4/A	PM15	Turkey	1993	11,12	3	7/R4	38/A	2	2	2	2	2	2	3
5/A	PM18	Chicken	1986	3	3	10/R2	22/A	3	3	3	3	4	2	4
6/A	PM19	Turkey	1986	3	3	5/R3	31/A	4	4	2	2	5	3	5
7/A	PM27	Chicken	1986	3	3	2/R5	19/A	2	5	4	4	5	4	6
	PM48	Chicken	1983	3,4	3	2/R5	18/A	2	5	4	4	5	4	6
	PM52	Chicken	1983	3,4,12	3	2/R5	20/A	2	5	4	4	5	4	6
	PM63	Chicken	1976	3	3	2/R5	1/A	2	5	4	4	5	4	6

ST & Cluster	Strain	Source	Year of Isolation	Somatic Serovar[A]	Biovar[B]	Ribotype & Cluster[C]	ET & Cluster (MLEE)[C]	Allelic profile						
								adk	*est*	*pmi*	*zwf*	*mdh*	*gdh*	*pgi*
8/A	PM35	Turkey	1986	3	3	5/R3	7/A	4	4	2	5	5	3	5
	PM40	Chicken	1988	3	3	5/R3	7/A	4	4	2	5	5	3	5
	PM45	Chicken	1986	3,4	3	5/R3	11/A	4	4	2	5	5	3	5
	PM75	Duck	1979	3,4,12,14	3	5/R3	29/A	4	4	2	5	5	3	5
9/A	PM37	Chicken	1988	3	1	12/R7	6/A	2	6	5	6	4	5	7
10/A	PM46	Chicken	1992	6	1	7/R4	3/A	5	7	6	7	1	6	8
11/A	PM49	Turkey	1984	1,15	1	14/R6	9/A	2	8	7	8	4	3	9
12/A	PM51	Chicken	1984	4,12	3	11/R5	13/A	6	9	5	9	6	3	8
13/A	PM55 (NCTC 10322)	Porcine	1962 Canada	NK	2	17/R6	R12/A	7	10	3	10	4	7	8
14/A	PM57 (NCTC 10204)	Bovine	1960 UK	NK	11	13/R7	R13/A	8	9	2	11	4	8	10
15/B	PM59 (CIP A125)	Human (Cat bite)	1952 France	NK	7	6/R1	R15/B	1	4	8	1	1	1	11
16/A	PM64	Chicken	1979	3	5	9/R8	16/A	9	11	9	12	5	8	12
17/A	PM65	Chicken	1979	4	5	9/R8	16/A	10	11	9	12	5	8	12
18/A	PM67	Turkey	1969	3,12	4	13/R7	14/A	11	5	10	8	4	4	8

ST & Cluster	Strain	Source	Year of Isolation	Somatic Serovar[A]	Biovar[B]	Ribotype & Cluster[C]	ET & Cluster (MLEE)[C]	Allelic profile						
								adk	*est*	*pmi*	*zwf*	*mdh*	*gdh*	*pgi*
19/A	PM69	Chicken	1973	NT	3	5/R3	32/A	11	4	2	5	5	3	5
	PM78	Chicken	1981	3	3	5/R3	29	11	4	2	5	5	3	5
	PM80	Chicken		3,4,12	3	5/R3	26/A	11	4	2	5	5	3	5
	PM86	Turkey	1988	3	3	5/R3	7/A	11	4	2	5	5	3	5
	PM95	Chicken	1991	3	3	5/R3	28/A	11	4	2	5	5	3	5
	PM136	Turkey	1992	3	3	5/R3	25/A	11	4	2	5	5	3	5
20/A	PM71	Chicken	1979	4	1	20/R5	5/A	12	12	11	4	5	8	6
21/B	PM72	Chicken	1977	3,14	6	4/R1	51/B	13	13	12	13	7	9	13
22/A	PM73	Turkey		4	3	2/R5	2/A	12	5	7	4	5	3	8
	PM132	Turkey	1992	4	3	2/R5	33/A	12	5	7	4	5	3	8
23/A	PM76	Chicken	1979	NT	5	9/R8	15/A	10	14	9	12	5	8	14
24/A	PM77	Chicken	1980	3	3	10/R2	21/A	14	3	3	3	4	2	4
25/A	PM79	Chicken		1	8	15/R2	39/A	11	15	2	14	8	10	14
26/B	PM81	Chicken		NT	6	4/R1	52/B	13	13	12	13	7	1	13
27/A	PM83	Chicken		NT	1	12/R7	4/A	12	6	13	6	4	5	7
28/A	PM84	Chicken		NT	1	12/R7	37/A	12	6	13	15	4	5	7
29/A	PM85	Turkey	1988	3	3	5/R3	7/A	11	4	2	5	5	3	15
30/A	PM87	Chicken	1986	3	3	5/R3	11/A	11	4	14	5	5	3	15

ST & Cluster	Strain	Source	Year of Isolation	Somatic Serovar[A]	Biovar[B]	Ribotype & Cluster[C]	ET & Cluster (MLEE)[C]	Allelic profile						
								adk	*est*	*pmi*	*zwf*	*mdh*	*gdh*	*pgi*
31/A	PM88	Chicken	1986	NT	1	3/R6	10/A	12	16	7	16	4	3	8
32/B	PM91	Chicken	1986	3	2	6/R1	44/B	15	1	1	1	1	1	1
33/A	PM96	Chicken	1991	3	4	13/R7	17/A	11	5	7	17	4	4	8
34/A	PM97	Turkey	1991	NT	3	11/R5	34/A	16	9	5	9	6	3	8
	PM120	Turkey		12	3	11/R5	12/A	16	9	5	9	6	3	8
35/B	PM131	Chicken		1	2	4/R1	55/B	15	17	15	8	9	1	11
36/A	PM133	Turkey	1992	4	3	1/R1	27/A	16	18	10	18	5	11	16
	PM137	Turkey	1992	4,7	3	1/R1	36/A	16	18	10	18	5	11	16
37/B	PM135	Turkey	1992	13	10	4/R1	47/B	17	19	13	5	1	1	5
38/B	PM138	Chicken	1994	1,15	2	4/R1	54/B	15	20	15	1	1	1	11
39/B	PM140	Chicken		13,14,15	7	4/R1	48/B	17	21	16	1	1	5	17

[A] As reported by Blackall *et al.* (1998). NT = Non-typable, NK = Not known.
[B] As reported by Blackall *et al.* (1998) using the biovars defined by Fegan *et al.* (1995)
[C] As established by Blackall *et al.* (1998)

Table 7. Properties of MLST Clades A and B.

MLST Clade	No. of Strains	No. of STs	Biovar/Species[A]	Serovar	Ribotype Cluster	MLEE Cluster
A	54	29	1 (6); 2 (2); 3 (39), 4 (2) / *multocida* (49)	1 (1)	R1 (2)	A (54)
				3 (18)	R2 (3)	
			5 (3) / ?? (3)	4 (7)	R3 (13)	
				6 (1)	R4 (14)	
			8 (1), 11(1) / *gallicida* (2)	10 (1)	R5 (10)	
				12 (1)	R7 (6)	
				Cross-reacting (12)	R6 (3)	
				Nontypable (7)	R8 (3)	
				Not known (2)		
B	12	10	2 (7) / *multocida* (7)	1 (1)	R1 (12)	B (12)
				3 (3)		
			6 (2) / ?? (2)	13 (1)C		
				Cross-reacting (5)		
			7 (2), 10 (1) / *septica* (3)	Nontypable (2)		
				Not known (1)		

[A] ?? = Biovars that could not be assigned to a recognised subspecies within *P. multocida* according to Fegan *et al.* (1995).

Chapter 3: Fowl cholera epidemiology and MLST typing

Introduction

A number of the existing methods for the typing of *P. multocida* – specifically REA, ribotyping, PFGE, REP-PCR and MLEE, have been used on the same set of 22 avian *P. multocida* obtained from outbreaks of fowl cholera on seven Australian turkey farms (Blackall & Miflin, 2000). Of these 22 isolates, a total of 21 have been examined in the current MLST study. The investigation of the relationships or otherwise among outbreaks of fowl cholera is a potential major application of MLST typing. Hence, this existing multiple application of typing methods to a single set of *P. multocida* isolates with a detailed field history is a good opportunity to directly compare MLST typing with a range of existing typing methods in a relevant setting.

Original epidemiological study – field information, biotyping, REA and ribotyping

The 22 isolates of *P. multocida* that form the basis of this evaluation have been subjected to extensive phenotypic characterisation, as well as REA and ribotyping using the enzyme *Hpa*II (Blackall *et al.*, 1995). The 22 isolates were obtained from 14 different birds on seven meat turkey farms. Based on the field information, these 22 isolates represented eight outbreaks. Multiple isolates were obtained from Farms 1 and 2 (five and 12 isolates, respectively), while only single isolates were available from Farms 3 to 7 (Blackall *et al.*, 1995). The five isolates from Farm 1 were obtained from four birds and the 12 isolates from Farm 2 were from six birds. A summary of the previously recorded results for biotyping, REA and ribotype of the 22 isolates is shown in Table 8.

MLEE Analysis

The same 22 isolates were subsequently part of the first population structure study for *P. multocida* (Blackall *et al.*, 1998). A direct evaluation of the use of MLEE was then performed (Blackall *et al.*, 1999). The MLEE typing results of the 22 isolates are shown in Table 8.

Pulsed Field Gel Electrophoresis (PFGE) and Repetitive Extragenic palindromic (REP) PCR

In a further study, Gunawardana *et al.* (2000) typed the same 22 isolates (as part of larger study on the typing of *P. multocida*) using two further techniques – PFGE and REP-PCR. The results of the PFGE and REP-PCR typing are also shown in Table 8.

Comparison of typing results

For Outbreaks II, VI, VII and VIII, all the typing methods agree that the outbreaks are distinct and not connected. All the typing methods agree that the two outbreaks seen on Farm 1 (Outbreaks I and II) are different. Four of the methods, biovar, REA, ribotyping, and MLST, indicate that Outbreaks I and V are connected with the outbreaks being associated with strains that are indistinguishable. By PFGE and REP PCR, the strains from Outbreaks I and V showed a slight difference (as indicated by the use of Roman numerals). These PFGE and REP-PCR differences were subtle and not sufficient to allocate the strains to different types. A similar slight difference was seen with MLEE – the two strains being allocated to different ETs that linked at a genetic distance of 0.25.

Five of the six molecular typing methods, REA, ribotyping, REP-PCR, MLEE and MLST, agree that Outbreaks III and IV are distinct and unconnected. However, PFGE typing suggested that the strains involved in these two outbreaks had identical types and thus were directly connected to a common strain.

Multiple isolates were examined within Outbreak III. By REA, ribotyping and MLEE, no variation was found within any of the 12 strains examined. PFGE and REP PCR identified a minor variation in PM 11 that was not detected by any other method. Similarly, MLST detected a minor difference in PM 15. This strain had a single nucleotide variation in the *pgi* locus and was thus allocated to a different ST (ST 4) than that for all the other strains of this outbreak (ST 2). Figure 2 (Chapter 2) indicates that these two STs are closely related (ST 2 is marked as Clade O) and join at a linkage distance of 0.15.

Overall performance of MLST typing as an epidemiological tool

The results of the MLST typing showed a good correlation with the other molecular typing methods. This evaluation will be set in two contexts – an ability to separate unrelated strains and an ability to link related isolates.

In terms of an ability to link related isolates, MLST performed strongly. MLST indicated that 11 of the 12 strains from Outbreak II all belonged to the same ST (ST 2). One strain (PM 15) was assigned to a different but closely related ST (ST 4). Two of the other molecular methods also detected minor differences in another strain associated with this outbreak – with PFGE and REP PCR both indicating that PM 11 was slightly different from the other strains.

Similarly, MLST confirmed all four isolates from Outbreak I were related – assigning three strains to the same ST (ST 1) and the fourth to a very similar ST (ST 3). ST 1 and ST 3 differ in only one allele (the *mdh* locus) and join at a linkage distance of 0.15 (see Clade V in Figure 2 in Chapter 2). The identity of the four strains involved in Outbreak I was confirmed by REA, ribotyping, PFGE and REP PCR. MLEE did detect a small difference in the three strains with one strain being assigned to ET 42 and the other two strains to ET 43. This difference is only minor as ET 42 and ET 43 join each other at a genetic distance of 0.05 (Blackall *et al.*, 1998).

MLST indicated that Outbreaks I and V were connected – with both Outbreaks being associated with the same ST (ST 36). The connection between these outbreaks was confirmed by REA and ribotyping which found the strains associated with the two outbreaks to be identical. PFGE and REP PCR typing also linked the two outbreaks – although minor differences were detected (but

not sufficient to assign to a different pattern type). MLEE typing assigned the Outbreak I strain to ET 36 and the Outbreak V strain to ET 35 – with these two ETs joining each other at a genetic distance of 0.25. Hence, while minor differences were detected by PFGE, REP PCR and MLEE, all typing methods essentially agree with the MLST result – Outbreaks I and V are connected and associated with the same strain.

In terms of an ability to separate un-related strains, MLST also performed well. MLST typing indicated that Outbreaks II, III, IV, VII and VIII were all distinctly different outbreaks with no sharing of strains between the outbreaks. This was the conclusion also reached by REA, ribotyping, REP PCR and MLEE. The only disagreement was that PFGE assigned the strain associated with Outbreak IV to the same pattern type as the strains associated with Outbreak II (PFGE type 20). As all other typing methods did not connect these outbreaks, it would appear that the PFGE result is the anomalous result.

Table 8. Comparison of typing methods using 22 avian isolates of *P. multocida*

Farm[A]	Outbreak[A]	Strains[A]	Date[A]	Subspecies[A]	Biovar[B]	REA Type[A]	Ribotype[A]	PFGE Type[C]	REP PCR Type[C]	MLEE[D]	MLST[E]
1	I	PM 137	10/92	*multocida*	3	I	i	19ii	20ii	36/A	36/A
	II	PM 1	2/93	*multocida*	2	VI	vi	4	4	42/B	1/B
		PM 12	3/93	*multocida*	2	VI	vi	4	4	43/B	1/B
		PM 13	3/93	*multocida*	2	VI	vi	4	4	43/B	1/B
		PM 14	3/93	*multocida*	2	VI	vi	4	4	43/B	3/B
2	III	PM 2	2/93	*multocida*	3	VII	vii	20	21	38/A	2/A
		PM 3	2/93	*multocida*	3	VII	vii	20	21	38/A	2/A
		PM 4	2/93	*multocida*	3	VII	vii	20	21	38/A	2/A
		PM 5	2/93	*multocida*	3	VII	vii	20	21	38/A	2/A
		PM 6	2/93	*multocida*	3	VII	vii	20	21	38/A	2/A
		PM 7	2/93	*multocida*	3	VII	vii	20	21	38/A	2/A
		PM 8	2/93	*multocida*	3	VII	vii	20	21	38/A	2/A
		PM 9	2/93	*multocida*	3	VII	vii	20	21	38/A	2/A
		PM 10	2/93	*multocida*	3	VII	vii	20	21	38/A	2/A
		PM 11	2/93	*multocida*	3	VII	vii	20ii	21ii	38/A	2/A
		PM 15	3/93	*multocida*	3	VII	vii	20	21	38/A	4/A
		PM 16	3/93	*multocida*	3	VII	vii	20	21	38/A	2/A
3	IV	PM 132	10/92	*multocida*	3	II	ii	20	22	33/A	22/A
4	V	PM 133	10/92	*multocida*	3	I	i	19i	20i	35/A	36/A
5	VI	PM 134	10/92	*multocida*	3	III	iii	18	19	27/A	ND
6	VII	PM 135	12/92	*septica*	10	IV	iv	17	17	47/B	37/B
7	VIII	PM 136	12/92	*multocida*	3	V	v	16	16	25/A	19/A

[A] As defined previously by Blackall *et al.* (1995).
[B] As defined previously by Blackall *et al.* (1995) and subsequently modified by Blackall *et al.* (1997).
[C] As defined previously by Gunawardana *et al.* (2000).
[D] As defined previously by Blackall *et al.* (1998).
[E] As defined in the previous Chapter of this Report.

Chapter 4: Discussion

The general need for a universal typing scheme for each species of pathogenic bacteria is widely acknowledged. As an example, Baldwin *et al.* (2005) have made the following statement "Schemes for the unequivocal typing and characterization of isolates are essential for epidemiological and evolutionary analysis of bacterial pathogens". To date, no such universal scheme has been developed for *P. multocida* (Blackall & Miflin, 2000).

MLST is a highly discriminatory and unambiguous method of characterizing bacterial isolates (King *et al.*, 2002) that has now been successfully employed in the typing of a range of pathogens (Dingle *et al.*, 2001; Enright & Spratt, 1998; Enright *et al.*, 2000; Enright *et al.*, 2001; Maiden *et al.*, 1998). MLST is based on the nucleotide sequences of internal fragments of housekeeping genes, in which mutations are assumed to be largely neutral (Selander *et al.*, 1986). Typically, seven distinct loci are used in the creation of an MLST scheme. Due to the high numbers of alleles at each of the seven loci it is highly unlikely that isolates will have the same profile by chance (King *et al.*, 2002). An important advantage of MLST is that sequence data are portable and can be readily compared among laboratories (King *et al.*, 2002). In addition, MLST data is useful beyond epidemiological applications, it can be used to address questions about the evolutionary and population biology of bacterial species (Feil *et al.*, 1999; Spratt, 1999).

Hence, this project was undertaken to develop the accepted gold standard of typing methods – MLST – for *P. multocida*. The study had two distinct advantages that assisted the development of the MLST scheme. The first advantage was that the genome of a strain of *P. multocida* has been fully sequenced (May *et al.*, 2001). This meant that the design of primers could be based on the known sequence of the selected gene loci within the sequenced *P. multocida* strain – strain PM70. The second advantage of this study was that an MLEE study has been completed in the same laboratory undertaking the current MLST study (Blackall *et al.*, 1998). This completed MLEE study meant that there was an existing knowledge of which house-keeping enzymes showed high diversity at the phenotypic level. As well, the availability of the strain collection used in the MLEE study for the current study meant that direct comparisons of the results of the MLST study with the MLEE study.

In performing this study, we used 66 strains of *P. multocida*. The number of strains used to create MLST schemes has varied widely. The MLST scheme for five clinically relevant serovars of *Salmonella enterica* (serovars Agona, Heidelberg, Schwarzengrund, Typhimurium and Typhimurium var Copenhagen) was created initially with 25 isolates (Sukhnanand *et al.*, 2005). MLST schemes for *Acinetobacter baumanii* (Bartual *et al.*, 2005), *Campylobacter coli* (Dingle *et al.*, 2005), *Klebsiella pneumoniae* (Diancourt *et al.*, 2005) and *Streptococcus uberis* (Zadoks *et al.*, 2005) have all been established with similar numbers of strains as was used in the current study. While some MLST schemes have been created using larger number of strains e.g. 143 isolates in the *Pseudomonas aeruginosa* scheme (Curran *et al.*, 2004) and 301 isolates in the *S. suis* (King *et al.*, 2002), our use of 66 strains was within the accepted range. In addition, the strains we used were deliberately selected from the prior MLEE study of Blackall *et al.* (Blackall *et al.*, 1998)

The primers are designed and optimised using two different methods in the two most recognised MLST models. In the *N. meningitidis* scheme (Maiden *et al.*, 1998) a nested PCR protocol was followed. In this approach one set of primers is used to amplify the target gene. A second set of primers, that target regions within the original sequence, are then used to amplify and sequence a 450-500 bp fragment. Alternatively, the *S. pneumoniae* scheme (Enright & Spratt, 1998) used the same primer set for both PCR and sequencing. The latter, a single set of primers for both original amplification and subsequent sequencing, was adopted in this study. Additionally most primers (seven of the eight pairs) were designed within a close Tm range, enabling the performance of multiple PCRs for several different genes simultaneously on the same PCR machine. This is a great

advantage when considering the time saving and equipment usage as well as simplifies the methodology.

The aim of a simplified PCR approach for this MLST was mostly achieved. We found that a single set of primers was sufficient to amplify six of the seven genes from all 66 strains examined. Single sets of primers for all gene loci involved in an MLST scheme is the preferred option and is a feature of MLST schemes for organisms such as *Ps. aeruginosa* (Curran *et al.*, 2004) and *S. suis* (King *et al.*, 2002). However, the requirement to use additional primer sets (as was found necessary for the *zwf* locus of *P. multocida*) has been also necessary in other MLST schemes for organisms such as *A. baumannii* (Bartual *et al.*, 2005) and *C. jejuni* (Manning *et al.*, 2003). We also originally aimed at using a single set of PCR amplification conditions for all seven gene loci – a common methodology in MLST schemes. We achieved this aim for six of the loci – with a different annealing temperature being necessary for the *gdh* locus. Other MLST schemes for organisms such as *Salmonella enterica* (Sukhnanand *et al.*, 2005), *S. suis* (King *et al.*, 2002) and *S. uberis* (Zadoks *et al.*, 2005) have encountered a similar need for variable PCR conditions.

The MLST study revealed that the *P. multocida* examined fell into two major clades (identified as A and B in Figure 2, Chapter 2). This structure matches closely the population structure recognised by both MLEE and ribotyping as reported previously (Blackall *et al.*, 1998). Indeed, the correlation of MLST with MLEE is very strong. A total of 13 strains from MLEE cluster B were examined in this study – with 12 of the 13 being assigned to MLST Clade B. The exception was strain PM 17 (a discussion on this apparent anomaly follows later). In the prior, ribotyping/MLEE study of Blackall *et al.* (1998), it was noted that Ribotype Cluster R1 was a close match for MLEE cluster B. This same correlation, i.e. Ribotype Cluster R1 and MLST Clade B, was repeated in the MLST study. More specifically, Blackall *et al.* (1998) reported that Ribotype Cluster R1 consisted of three ribotype patterns – patterns 1, 4 and 6. In this MLST study, 12 strains (six of ribotype patter 4 and six of ribotype pattern 6) were examined. All 12 strains were allocated as the only members of MLST Clade B. This matches the prior study for MLEE and ribotyping – with the exception again of strain PM 17. In the MLEE/ribotyping study, PM 17 was the exception – being the only strain allocated to MLEE Cluster B that was not of ribotype patterns 1 or 4.

This MLST study confirms the unique position of strains within MLST Clade B. There is now evidence from ribotyping, MLEE and MLST that strains within MLST Clade B are quite distinct and separate from other strains of *P. multocida*. As noted earlier by Blackall *et al.* (1998), strains within MLST Clade B (i.e. MLEE Cluster B) represent two subspecies (*multocida* and *septica*), show unusual biochemical properties and are notable for lacking strains belonging to the dominant biovar of *P. multocida* – biovar 3. It is now quite clear that the strains of MLST Clade B have a common genetic background that is quite distinct from the other strains of *P. multocida* examined by MLST and MLEE to date. This sub-grouping by MLST cuts across the currently recognised sub-species within *P. multocida* (*gallicida*, *multocida* and *septica*). It is also notable that there seems to be no association with somatic serovar and MLST Clade B. This is further evidence that the underlying population structure of *P. multocida* has little to do with somatic serovars – a finding also noted in the MLEE/ribotyping study (Blackall *et al.*, 1998).

Of the 66 strains examined in both the MLST study reported here and the previous MLEE/ribotyping study of Blackall *et al.* (1998), only one strain showed a markedly different allocation – strain PM 17. In the MLEE study, PM 17 was assigned to ET 45 within MLEE Cluster B. In contrast, MLST analysis assigned PM 17 to ST 2 within MLST Clade A. There are a number of pieces of evidence that the MLST placement is correct while the MLEE placement is erroneous. PM 17 is from the same turkey farm as isolates PM 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 15 and 16. All the isolates were obtained at the same time in 1993 in an outbreak of fowl cholera. As shown in Table 6 (Chapter 2), MLST assigned isolates PM 2- 11 and PM 16 and 17 to a single ST – ST 2. Strain PM 15 was assigned to a closely related ST (ST 4) that joined at a linkage distance of 0.15 and differed by only a single nucleotide change in the *pgi* locus (Table 6, Chapter 2). Ribotyping agreed with MLST – placing PM 2 -11 and PM 15-17 in ribotype 7. Hence, the known epidemiology (a confined outbreak on a single farm) and

the ribotyping results all support the conclusion of the MLST study i.e. that PM 17 should be aligned with strains PM 2 – 11 and PM 15 and 16.

According to Ogle *et al.* (1987), an epidemiological marker should 1) be sufficiently sensitive to distinguish all unrelated isolates, 2) specifically identify all related isolates and 3) be stable. We have used these same criteria – sensitivity, specificity and stability (all in an epidemiological sense) – to evaluate MLST typing in the investigation of fowl cholera outbreaks. As the examination was a retrospective study involving a series of well-characterised outbreaks (in terms of field and laboratory investigations), we were able to compare MLST with almost all forms of commonly used molecular typing methods for *P. multocida*. This comparison (fully detailed in Chapter 3) indicates that MLST typing is sensitive (separates unrelated strains), specific (links related strains) and is stable (only one minor allele change of a single nucleotide was detected amongst twelve isolates from a single outbreak). Similar high specificity, sensitivity and stability were demonstrated by the other molecular methods (REA, ribotyping, PFGE and REP PCR). The distinct advantage of MLST is portability and an ability to compare strains and results across the continents. The creation of the MLST scheme for *P. multocida* represents a major step forward in the ability of scientists around the world to type and compare isolates of *P. multocida* from all hosts.

The data-base generated in this study is currently being placed on the Oxford University MLST Website (http:/pubmlst.org/software/analyis/). The data will then be publicly available, allowing the scheme as it exists to be used by others and most importantly allowing others to add to the data-base.

A notable feature of the MLST study was the placement of the three taxonomic reference strains in clades that contained no other strain and which were quite distinct from all other strains. This was particularly noticeable with the reference strain for the subspecies *gallicida* which did not join a clade containing Australian isolates till a linkage distance of 0.85. The reference strain for subspecies *multocida* did not join an Australian strain till a linkage distance of 0.7. The subspecies *septica* was the most closely linked – joining with an Australian strain at a distance of 0.45.

These results for the three reference strains in MLST match the results from the previous MLEE study (Blackall *et al.*, 1998). In the MLEE study, the reference strains for *multocida* and *gallicida* first joined with each other before joining any Australian strain (at a genetic distance of 0.4). As in the MLST study, the MLEE study found that the *septica* reference strain showed a closer linkage with the Australian strains – joining at a genetic distance of 0.35.

It is possible that the marked distinction between the reference strains and the Australian strains found by both MLST and MLEE may be associated with host source. All the Australian strains in the MLST and MLEE studies were from poultry. In contrast, the reference strains are from a pig (*multocida*), a cow (*gallicida*) and a human suffering from a cat bite wound (*septica*). There have been suggestions that *P. multocida* might represent different species and that the host-parasite relationship may be part of this differentiation within *P. multocida* (Petersen *et al.*, 2001). An alternative explanation for the relative distance between the reference strains and the Australian isolates may be geographical (the reference strains come from Canada, the UK and France) or time (the reference strains were all isolated in the 1950s and 1960s). Clearly, further work is required. As other research groups add to the MLST data-base, it should be possible to identify if geography, host and/or time play a role in the overall population structure of *P. multocida*.

This study represents the first formal proposal of an MLST scheme for *P. multocida*. However, there have been two previous studies that have looked at the sequences of house-keeping genes (Christensen *et al.*, 2005; Davies *et al.*, 2004). Davies *et al.* (2004) examined 36 bovine isolates using seven house-keeping enzymes. This bovine study did not utilise the conventional MLST analysis techniques – rather the seven sequences for each isolate were concatenated into a single sequence of 3,990 nucleotides and a minimum evolution tree was created. Davies *et al.* (2004) concluded on the basis of this analysis that the method did not achieve a high level of strain differentiation and that outer membrane typing was more discriminatory. As the current study is a full MLST study, we have

used very different analysis techniques. In addition, the current study was focussed on poultry isolates of *P. multocida*. The two studies had four house-keeping genes in common and three that were unique to each study. Hence, it is difficult to compare the results of the current study with the study of Davies *et al.* (2004).

The study of Christensen *et al.* (2005) involved the analysis of the gene sequences of two house-keeping genes (*rpoB* and *infB*) for taxonomic and phylogenetic purposes. Christensen *et al.* (2005) reported that the analysis of these two genes showed a good correlation with each other and that the analysis provided a deeper resolution at the species level than achieved by analysis of the gene typically used for species resolution – the 16S rRNA gene.

In conclusion, this study represents the first time an MLST scheme has been established for *P. multocida*. The study provided further confirmation that the *P. multocida* population consists of two quite distinct clades. The study also confirmed that MLST typing is highly specific, sensitive and stable (in an epidemiological sense) and performs as well as any other available typing method. As MLST is totally portable and easily comparable across laboratories, we suggest that MLST should now be accepted as the "gold standard" typing method for *P. multocida*. The availability of the data-base generated in this project at the public MLST Website (http:/pubmlst.org/software/analyis/) will encourage the adoption and extension of this scheme by other laboratories around the world.

Implications

In this project we have developed the first ever MLST typing scheme for *P. multocida*. The application of the MLST scheme has confirmed that Australian poultry isolates of *P. multocida* form two very distinct sub-populations. A detailed evaluation demonstrated that MLST is also a very powerful tool for investigating outbreaks of fowl cholera. Overall, this work has produced a technology that will greatly assist our understanding of the basic biology of *P. multocida* plus provide new insights into how and why disease outbreaks of fowl cholera spread. The MLST scheme developed in this study will prove a long-term, valuable investment that will underpin improved and sustainable methods for the control of fowl cholera and a range of the other diseases in other hosts caused by *P. multocida*. The Microbiology Research Group will provide MLST typing of *P. multocida* isolates on a "user pays" basis to the Australian poultry industry.

Recommendations

This research project has resulted in the first creation of an MLST scheme for *P. multocida*. The adoption, by the Australian poultry industry of MLST, as a typing tool for other important bacterial pathogens of chickens as well as bacterial zoonotic agents should be encouraged, where relevant.

The Microbiology Research Group has undertaken to provide MLST typing of *P. multocida* on a "user pays" basis. This commercial service will allow an on-going access to this technology. The service will supplement other specialised reference services (all developed with poultry industry funding) that the Microbiology Research Group currently provides to the poultry industry.

A future improvement to the *P. multocida* MLST scheme could include the use of single nucleotide polymorphism (SNP) analysis as opposed to full DNA sequencing. The use of SNP analysis has already been proposed for other MLST schemes e.g. the *Campylobacter* scheme (Best *et al.*, 2004). While not capable of recognising new STs, the SNP approach does allow a very rapid and very cost effective typing within an existing MLST scheme (Best *et al.*, 2004).

Communications strategy

Preliminary results from this work have been presented at one international poultry health conference. The details of this conference are as follows:-

Subaaharan, S., Blackall, L.L. and Blackall, P.J. (2004) Towards an MLST scheme for *Pasteurella multocida*. 5th Asia Pacific Poultry Health Conference, Gold Coast.

The placement of the data-base generated by this project at the MLST Website (http:/pubmlst.org/software/analyis/) will encourage the adoption and extension of this scheme by other laboratories.

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