RURAL INDUSTRIES RESEARCH
& DEVELOPMENT CORPORATION

Diagnosing Avian Respiratory Diseases

Development of diagnostic tools for the rapid and specific detection of avian bacterial pathogens

A report for the Rural Industries Research and Development Corporation

by Jeanette Miflin and Pat Blackall

October 1998

RIRDC Publication No 98/105
RIRDC Project No DAQ-200A(J)

© 1998 Rural Industries Research and Development Corporation.
All rights reserved.

ISBN 0 642 57831 1
ISSN 1440-6845

"Diagnosing Avian Respiratory Diseases – Development of diagnostic tools for the rapid and specific detection of avian bacterial pathogens"
RIRDC Publication no. 98/105
RIRDC Project no. DAQ-200A(J)

The views expressed and the conclusions reached in this publication are those of the author and not necessarily those of persons consulted. RIRDC shall not be responsible in any way whatsoever to any person who relies in whole or in part on the contents of this report.

This publication is copyright. However, RIRDC encourages wide dissemination of its research, providing the Corporation is clearly acknowledged. For any other enquiries concerning reproduction, contact the Communications Manager on phone 02 6272 3186.

Researcher Contact Details

Name: Dr Pat Blackall
Organisation: Queensland Department of Primary Industries
Queensland Poultry Research and Development Centre
Animal Research Institute
665 Fairfield Road
YEERONGPILLY QLD 4105

Locked Bag 4
MOOROOKA QLD 4105

Phone: 07 33629498
Fax: 07 33629429
email: blackap@dpi.qld.gov.au

RIRDC Contact Details

Rural Industries Research and Development Corporation
Level 1, AMA House
42 Macquarie Street
BARTON ACT 2600

PO Box 4776
KINGSTON ACT 2604

Phone: 02 6272 4539
Fax: 02 6272 5877
email: rirdc@netinfo.com.au
Internet: http://www.rirdc.gov.au

Published in October 1998
Printed on environmentally friendly paper by the DPIE Copy Centre

Foreword

Respiratory diseases are a major cause of economic losses to the Australian chicken meat and egg industries. Two bacterial diseases that are important contributors to the respiratory disease complex are fowl cholera and infectious coryza.

Both of these diseases present considerable challenges for diagnostic laboratories. *Pasteurella multocida*, the causative agent of fowl cholera, is a member of a very extensive family of closely related organisms, many of which have only recently been recognised and are difficult to distinguish. *Haemophilus paragallinarum*, the causative agent of infectious coryza, is difficult to grow in the laboratory and difficult to identify.

There is a need for new, rapid and accurate diagnostic tools for both of these diseases. The polymerase chain reaction (PCR), which detects signature DNA sequences, offers the potential for a rapid, specific and sensitive diagnostic tool.

This publication describes the development and validation of PCR tests for *Pasteurella multocida* and *Haemophilus paragallinarum*. The work reported here established that these PCR tests offer considerable advantages over the traditional diagnostic method of culture.

The report forms part of RIRDC's Chicken Meat R&D program, which aims to support increased profitability and sustainability in the chicken meat industry through improvements in efficiency, product quality and market size and through the adoption of enlightened management practices.

Peter Core
Managing Director
Rural Industries Research and Development Corporation

Acknowledgements

The authors would like to thank Dr Rod Jenner, Dr Peter Groves, Ms Anne Marie Hector and Ms Tina Stathos for their co-operation in this study.

Contents

Foreword .... *iii*

Acknowledgements .... *iv*

Executive Summary .... *vii*

1. INTRODUCTION 1

2. OBJECTIVES 3

3. DEVELOPMENT OF A PCR TEST FOR *PASTEURELLA MULTOCIDA* 4

4. VALIDATION OF TWO PCR TESTS FOR *HAEMOPHILUS PARAGALLINARUM* 15

5. IMPLICATIONS 26

6. RECOMMENDATIONS 27

7. INTELLECTUAL PROPERTY 28

8. COMMUNICATION STRATEGY 29

9. REFERENCES 30

Executive Summary

Respiratory diseases are a major cause of economic losses to the Australian chicken meat and egg industries. Two bacterial diseases that are important contributors to the respiratory disease complex are fowl cholera and infectious coryza.

Fowl cholera is a common and widely distributed disease of poultry which has major economic importance. The disease is caused by the bacterium *Pasteurella multocida*.

P. multocida has been extensively studied since it was first isolated in the late 1870s. In recent times, it has been recognised that there are three subspecies within *P. multocida* - *P. multocida* subsp. *multocida*, *P. multocida* subsp. *septica* and *P. multocida* subsp. *gallicida*. As well, another three new *Pasteurella* species (*P. anatis, P. langaa* and an un-named taxon termed *Pasteurella* species A) have recently been recognised in poultry - and all three resemble *P. multocida*. Hence, the members of the genus *Pasteurella* that have been formally recognised as being present in poultry are *P. anatipestifer* (also known as *Riemerella anatipestifer), P. anatis, P. avium, P. gallinarum, P. haemolytica* (also known as *Actinobacillus salpingitidis), P. langaa, P. multocida* (existing in three subspecies), *P. volantium* and *Pasteurella* species A. Hence, the task of confidently identifying a suspect *P. multocida* requires an extensive range of phenotypic testing that is beyond the capability of routine diagnostic laboratories.

Infectious coryza is an upper respiratory tract disease of chickens caused by the bacterium *Haemophilus paragallinarum*. The economic impact of the disease is mainly associated with a significant reduction (10%-40%) in egg production, especially on multi-age farms.

The traditional method of confirming infectious coryza in a chicken flock is the isolation and biochemical characterisation of *H. paragallinarum*. This is a technically demanding task as most *H. paragallinarum* isolates have stringent nutritional requirements, so that the normal media used in bacteriology laboratories does not support the growth of these organisms. This means that specialised media and techniques must be used to isolate *H. paragallinarum*. In addition, as *H. paragallinarum* is a slow growing organism, it is highly likely that commensal bacteria, which are typically vigorous growers, will mask the presence of the small colonies of *H. paragallinarum*. The diagnostic task is complicated by the occurrence in chickens of non-disease-causing bacteria that have similar nutritional requirements. These non-pathogenic bacteria can only be confidently distinguished from *H. paragallinarum* by means of extensive testing.

Clearly, both fowl cholera and infectious coryza present considerable challenges for diagnostic laboratories. There is a need for new, rapid and accurate diagnostic tools for these diseases.

The objective of the first part of the project was to develop a rapid and accurate molecular method for the detection of *P. multocida*.

The PM 23-2 PCR test developed in this project has been shown to be capable of detecting all three subspecies: *P. multocida* subsp. *multocida*, *P. multocida* subsp. *gallicida* and *P. multocida* subsp. *septica*.
The PM 23-2 PCR test also correctly identified 18 overseas serovar reference strains, indicating that it would be capable of detecting a wide variety of *P. multocida* strains. The

robustness of the PCR is indicated by the fact that these overseas reference strains have been collected from at least seven different animal hosts. The applicability of the PM 23-2 PCR was further demonstrated by the correct identification of 87 Australian avian field isolates, representing a collection with geographic, temporal and genetic diversity. Among this collection were two field isolates of subsp. *septica* and three of subsp. *gallicida*.

In addition, 36 porcine isolates were positive, suggesting that the PCR might be applicable to the diagnosis of porcine pasteurellosis. Worldwide, porcine pasteurellosis remains one of the most important diseases associated with intensive pig production. Hence the PM 23-2 PCR should be of considerable benefit to the pig industry (both in Australia and overseas) as well as to the diagnostic laboratories that serve the pig industry.

Of the 30 other bacterial species or subspecies tested in the PM 23-2 PCR, all were negative except for *P. avium* biovar 2 and *P. canis* biovar 2. As neither *P. avium* biovar 2 nor *P. canis* biovar 2 occur in poultry or pigs, *P. multocida* is the only organism of avian or porcine origin shown to give a positive reaction in the PM 23-2 PCR.

The conventional method for the identification of a suspect isolate as *P. multocida* requires that the isolate be subjected to an extensive range of biochemical tests. The PCR test has significant advantages over this conventional approach. The PM 23-2 PCR enables very rapid confirmation of a suspect colony. Indeed, the PCR test can give results in less than five hours, compared with two to five days for the conventional approach. As well, the identification given by the PM 23-2 PCR has been shown in this project to be very specific.

In summary, the PM 23-2 PCR represents a considerable step forward in our ability to diagnose fowl cholera. The PCR is specific and rapid. The equipment and expertise required to perform PCR-based tests are now widely available in the central government laboratories that serve the Australian poultry industries. Hence, this assay should be capable of being made rapidly available to the Australian poultry industries.

Recently, the diagnostic options available for infectious coryza have been expanded by the availability of a species-specific, rapid, DNA-based test for the identification of *H. paragallinarum* which is based on the polymerase chain reaction (PCR). This test, termed the HP-2 PCR, was developed and validated at the Animal Research Institute using artificially infected chickens in small scale pen trials. Subsequently, a second PCR test, named the 23S HP-PCR, has been developed at the Animal Research Institute. Neither test had been validated in commercial chickens, and this formed the second objective of the current project.

In the current study, we first demonstrated that the 23S HP-PCR is as effective as culture when used on mucus samples taken from live birds that have been artificially challenged with *H. paragallinarum*.

In order to evaluate the 23S HP-PCR and the HP-2 PCR tests with more realistic samples than those obtained from artificially infected chickens, two pen trials that imitated natural exposure were performed. In both trials, provided the mucus samples were tested on the day of collection, both the 23S HP-PCR and the HP-2 PCR were shown to be the equivalent of culture.
In one of the two natural exposure trials, we evaluated the impact of storage/transport on the results given by both PCRs and traditional culture. After 72 hours of simulated transport in a chilled esky, there were significantly more positive results in both PCR tests than in culture.

Clearly, PCR is a more robust technique than culture. This is an important finding as many diagnostic laboratories are likely to be attempting to diagnose infectious coryza by culturing swabs transported to a central laboratory. Our work has shown that culture can only be a reliable technique when used on freshly obtained swabs. In contrast, the two PCR techniques can be used on samples that have been delayed for up to 72 hours after collection.

The pen trials demonstrated that the two PCR tests perform at least as well as culture. However, there is a distinct advantage to the PCR tests - rapidity of results. PCR results are available within six to eight hours while confirmed culture requires three to five days.

When comparing the two PCR tests and traditional culture using samples from three outbreaks of infectious coryza in commercial chickens, the PCR tests were far superior. Indeed, traditional culture failed to yield a single pure culture of *H. paragallinarum* from two of the three outbreaks of coryza investigated. This was despite that fact that, in all three cases, the typical clinical signs of coryza were present and culture was performed in a very experienced laboratory. Indeed, it was only the PCR techniques, applied either directly to the living bird or to highly overgrown plate cultures, that allowed the confirmed diagnosis of infectious coryza to be made. If the three field investigations had been dependent upon traditional culture, none of the outbreaks would have been confirmed as coryza, despite the presence of the typical clinical signs and the field veterinarian's provisional diagnosis of coryza.

In the current study, we were unable to modify the 23S HP-PCR test to remove the low level of false positive reactions that the test is known to give. Hence, at the current state of development, the 23S HP-PCR for *H. paragallinarum* cannot yet be recommended as a replacement for traditional culture. Further refinement of the assay is needed.

The HP-2 PCR has been shown to give no false positive reactions. The current study has demonstrated that the HP-2 PCR is clearly superior to traditional culture when used on samples obtained from outbreaks of coryza in commercial chickens. The HP-2 PCR is strongly recommended for adoption, either as a total replacement for culture or as an additional test, for the diagnosis of infectious coryza.

In conclusion, the PM 23-2 PCR test for *P. multocida* and the HP-2 PCR test for *H. paragallinarum* described in this report can be recommended as either replacements for traditional culture-based methods or as supporting tests to be used in conjunction with culture. The PCR tests have been thoroughly validated, provide more rapid results than traditional methods and are cost-effective.

Introduction

Respiratory diseases are a major cause of economic losses to the Australian chicken meat and egg industries. Two bacterial diseases that are important contributors to the respiratory disease complex are fowl cholera and infectious coryza.

Fowl cholera is a common and widely distributed disease of poultry which has major economic importance (Rhoades *et al.* 1989). The disease is caused by the bacterium *Pasteurella multocida* (Rhoades *et al.* 1989).

Fowl cholera was first definitively recognised in Australia by Hart (1938) and has been reported in all Australian States (Beveridge and Hart 1985). It has been recorded in surveys of disease in broiler breeders, broilers and laying hens (Jackson *et al.* 1972; Grimes 1975; 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.

P. multocida has been extensively studied since it was first isolated in the late 1870s (Rhoades and Rimler 1991). In recent times, the application of new technologies has improved our knowledge of the organism. In particular, Mutters *et al.* (1985a) performed an extensive study using DNA homology and recognised 3 subspecies within *P. multocida* - *P. multocida* subsp. *multocida*, *P. multocida* subsp. *septica* and *P. multocida* subsp. *gallicida*. As well, Mutters *et al.* (1985a, b) described another three new *Pasteurella* species - *P. anatis, P. langaa* and an un-named taxon termed *Pasteurella* species A - which are present in avian hosts and which resemble *P. multocida*. Hence, the members of the genus *Pasteurella* that have been formally recognised as being present in poultry are *P. anatipestifer* (also known as *Riemerella anatipestifer), P. anatis, P. avium, P. gallinarum, P. haemolytica* (also known as *Actinobacillus salpingitidis), P. langaa, P. multocida* (existing in three subspecies), *P. volantium* and *Pasteurella* species A. Hence, the task of confidently identifying a suspect *P. multocida* requires an extensive range of phenotypic testing that is beyond the capability of routine diagnostic laboratories.

Infectious coryza is an upper respiratory tract disease of chickens caused by the bacterium *Haemophilus paragallinarum* (Blackall *et al.* 1997). The economic impact of the disease is mainly associated with a significant reduction (10%-40%) in egg production, especially on multi-age farms (Blackall *et al.* 1997).

While the disease is traditionally regarded as a mild disease, it is important to note that overseas countries have been reporting significant changes in the forms and severity of infectious coryza outbreaks. There has been a recent emergence of the disease in meat chickens in the USA, a situation not seen for over 30 years (Droual *et al.* 1990; Hoerr *et al.* 1994). In the Americas, apparently new forms of the disease have emerged. A swollen head-like syndrome associated with *H. paragallinarum* has been reported in North and South America (Droual *et al.* 1990; Sandoval *et al.* 1994). Arthritis and septicaemia, possibly complicated by the presence of other pathogens, have been reported in broiler and layer flocks in South America (Sandoval *et al.* 1994). In South Africa, Horner *et al.* (1995) have reported an increasing incidence of air-sacculitis associated with infectious coryza outbreaks.

Even in Australia, the classic, upper respiratory disease can be of significant economic impact. Arzey (1987) reported that, in a high health flock given prompt antibiotic treatment, an infectious coryza outbreak still caused an 11.4% drop in egg production over a six week period.

The traditional means of confirming infectious coryza in a chicken flock is the isolation and biochemical characterisation of *H. paragallinarum* (Blackall *et al.* 1997). This is a technically demanding task as most *H. paragallinarum* isolates show a requirement for nicotinamide adenine dinucleotide (NAD) for growth in artificial media (Blackall *et al.* 1997). As *H. paragallinarum* is a slow growing organism, there is a high possibility that commensal bacteria, which are typically vigorous growers, can mask the presence of the small colonies of *H. paragallinarum*. The diagnostic task is complicated by the occurrence of similar, but non-pathogenic, NAD-dependent bacteria, collectively known as the "*H. avium*" group (Blackall *et al.* 1997), in chickens. These organisms are now assigned to three species in the genus *Pasteurella* as *P. avium*, *P. volantium* and *Pasteurella* species A (Mutters *et al.* 1985b). These non-pathogenic bacteria can only be confidently distinguished from *H. paragallinarum* by means of extensive phenotypic testing.

Hence, diagnostic laboratories must first isolate a delicate, slow growing, nutritionally demanding organism. This requires the use of specialised media and/or isolation techniques. Suspect organisms must be then purified and subject to extensive phenotypic testing - again requiring specialised media and techniques.

Recently, the diagnostic options for infectious coryza have been expanded by the availability of a species-specific, rapid, PCR-based test for the identification of *H. paragallinarum* (Chen *et al.* 1996). This test, termed the HP-2 PCR, was developed and validated at the Animal Research Institute in artificially infected chickens in small scale pen trials (Chen *et al.* 1996). Subsequently, a second test, named the 23S HP-PCR, has been developed at ARI (Miflin, unpublished data). These potentially useful diagnostic tests had not been validated under realistic conditions typical of those occurring in commercial chickens suffering from infectious coryza.

In summary, fowl cholera and infectious coryza present considerable challenges for diagnostic laboratories. Fowl cholera is caused by a bacterium that is part of a very extensive genus of closely related organisms, many of which have only been recently recognised and are of uncertain pathogenic potential. Infectious coryza is caused by a bacterium that is difficult to grow, difficult to identify and which closely resembles a collection of normal, non-pathogenic bacteria. There is a need for new, rapid and accurate diagnostic tools for these diseases.

Both the chicken meat and egg industries have a goal of maximising production by minimising the effects of infectious diseases on flock health. This goal requires the ability to rapidly and accurately diagnose disease outbreaks. Indeed, the Australian industries have reached a level of sophistication where such rapid and accurate disease diagnosis is essential to maintain economic viability. The aim of this project was to provide diagnostic tests to meet those needs.

Objectives

The aim of this project was to develop and evaluate novel DNA based test methods to achieve rapid and accurate disease diagnosis for fowl cholera and infectious coryza.

The project had two major objectives. The first was to develop a rapid and accurate molecular method for the detection of *Pasteurella multocida*, the causative agent of fowl cholera. The second was to validate, using natural outbreaks of infectious coryza, a polymerase chain reaction (PCR) method for *Haemophilus paragallinarum* that had been developed in a project funded by the Australian Centre for International Agricultural Research (ACIAR). During the course of the current project, a second PCR test for *H. paragallinarum* was included in these evaluations.

Objective 1

Development of a PCR test for *P. multocida*

1.1 Introduction and explanation of methods

The polymerase chain reaction (PCR) has been applied to a wide range of human and animal diseases of bacterial, viral and parasitic origin. PCR has several major advantages that have contributed to its widespread adoption as a diagnostic technique. It is highly specific, enabling the identification of a single species from a mixed population. PCR is also very sensitive, being able in some cases to detect the presence of a single bacterial cell, or less than one millionth of one milligram of bacterial DNA. It is also very rapid, reducing the time required for infectious disease diagnosis from several days to several hours.

The essential element in the development of a PCR test is the identification of suitable species-specific oligonucleotide primers. Both 16S and 23S ribosomal RNA genes are suitable sources for PCR primers because there are conserved and variable regions in each molecule. This makes it possible to design oligonucleotide primers which are complementary to sequences that are specific for any level of the phylogenetic tree from kingdom to species (Wilson *et al*. 1990).

Following primer selection, the key parameters for the PCR such as magnesium chloride concentration, primer concentration and annealing temperature are optimised using the type strain of the target organism. The specificity of the primers is then determined by screening a range of type strains of related organisms. If these results look promising, the ability of the PCR to detect a wide variety of field strains of the target organism is determined. Further testing of field strains of other organisms is also desirable.

1.2 Methodology

1.2.1 Common procedures

1.2.1.1 Bacteria

Reference isolates of *P. multocida*

The type strains for the three subspecies of *P. multocida* were used in the initial development of the PCR tests:

P. multocida subsp. *multocida* NCTC 10322
P. multocida subsp. *gallicida* NCTC 10204
P. multocida subsp. *septica* CIP A125.

In addition, 16 somatic reference strains for the Heddleston typing scheme (Heddleston *et al.* 1972) and two reference strains for the Carter typing scheme (Carter 1955) were included in the study.

Avian field isolates of *P. multocida*

The study included a total of 87 field isolates collected from poultry from the eastern States of Australia between 1966 and 1997. These isolates consisted of 34 from turkeys, 35 from chickens, one from a duck and 17 from unspecified poultry. Among this collection were two isolates of subsp. *septica* and three isolates of subsp. *gallicida*, the remainder being subsp. *multocida*. This collection of isolates included representatives of the whole spectrum of genetic diversity of Australian avian *P. multocida* isolates recognised in RIRDC-funded project DAQ27CM (Blackall *et al*. 1996).

Porcine field isolates of *P. multocida*

The study included 36 Australian porcine *P. multocida* isolates.

Table 1. List of 12 members of the family *Pasteurellaceae* used in initial screening

Organism	Code Number
Actinobacillus salpingitidis	CCUG 23139
Haemophilus paragallinarum	ATCC 29545
Pasteurella anatis	NCTC 11413
Pasteurella avium	ATCC 29546
Pasteurella canis biovar 1	NCTC 11621
Pasteurella dagmatis	NCTC 11617
Pasteurella gallinarum	ATCC 13361
Pasteurella langaa	NCTC 11411
Pasteurella species A	HIM 789-5
Pasteurella species B	CCUG 19794
Pasteurella stomatis	NCTC 11623
Pasteurella volantium	NCTC 3438

Table 2. List of additional members of the family *Pasteurellaceae* used in the study

Organism	Code Number
Actinobacillus pleuropneumoniae	ATCC 27088
Actinobacillus pleuropneumoniae	HIM 677-34
Actinobacillus suis	CCUG 11624
Haemophilus Taxon C	CAPM 5111
Pasteurella avium biovar 2	CCUG 16497
Pasteurella canis biovar 2	HIM 843-5
Pasteurella haemolytica	CCUG 408
Pasteurella trehalosi	CCUG 27190

Table 3. List of additional type strains used in the study

Organism	Code Number
Alcaligenes faecalis	ATCC 8750
Bordetella avium	ATCC 35086
Bordetella bronchiseptica	ATCC 19395
Bordetella hinzii	LMG 13501
Ornithobacterium rhinotracheale	CCUG 23171

Field isolates of organisms other than *P. multocida* used in the study

Field isolates of a range of organisms were used in the specificity testing. These organisms and the number of strains tested for each one are listed below:
A. salpingitidis (6), *H. paragallinarum* (5), *Haemophilus parasuis* (2), *H.* Taxon C (1), *P. avium* (2), *P. gallinarum* (6), *Pasteurella haemolytica* (bovine) (1), *B. avium* (5), *B. hinzii* (5), *Escherichia coli* (1), *Staphylococcus aureus* (1), *Staphylococcus hyicus* (1), *Streptococcus suis* (1).

1.2.1.2 DNA extraction

Bacterial growth from an overnight blood agar plate was suspended in 500 µl SE buffer (150 mM NaCl, 100 mM di-sodium EDTA, pH 8.0). After the addition of 50 µl lysozyme (20 mg/ml) the suspension was incubated at 37°C for 1 h. Ten µl sodium dodecyl sulphate (25%) and 25 µl proteinase K (20 mg/ml) were added and the suspension incubated for 3 h at 56°C. The suspension was extracted sequentially with equal volumes of phenol; 1:1 phenol-chloroform/isoamyl alcohol (24:1) and chloroform/isoamyl alcohol (24:1). After the addition of 20 µl RNase (10 mg/ml) to the final supernatant it was incubated at 37°C for 1 h. The sequential phenol chloroform extractions were repeated and DNA precipitated from the final supernatant by using sodium acetate and ice-cold ethanol. The DNA pellet was rinsed in 70% ethanol and freeze-dried. The pellet was resuspended in sterile distilled water and the quality and concentration of the DNA determined spectrophotometrically. The quality of the DNA was also checked visually after agarose gel electrophoresis.

1.2.1.3 Standard PCR test

The standard PCR test consisted of a 50 µl reaction mixture containing x mM $MgCl_2$, 50 mM KCl, 10 mM Tris HCl (pH 8.3), 100 µg/mL gelatin, 200 µM each dNTP (dATP, dCTP, dGTP, dTTP), y µM each primer and 1.25 units *Taq* DNA polymerase (Boehringer Mannheim, Mannheim, Germany). The concentrations x and y were determined by optimisation experiments. The appropriate template was added: approximately 100 ng DNA or 1 µl of colony preparation (see below).

The PCR was performed using a Hybaid OmniGene Thermal Cycler (Hybaid, Middlesex, UK). An initial heating step at 98°C for 2.5 min was performed prior to the addition of the *Taq* DNA polymerase. A 50 µl mineral oil overlay was then added. The usual cycling parameters were: denaturing at 94°C for 1 min, annealing at T°C for 1 min and extension at 72°C for 1 min, these three steps being repeated for 30 cycles. This was followed by a final cycle with an extension step of 10 min. The annealing temperature T°C was determined by optimisation experiments to give the best specificity.

A positive control containing template DNA from *P. multocida* NCTC 10322 and a negative control containing no DNA were included in each assay run.

PCR products were detected by horizontal gel electrophoresis of a 10 µl aliquot in a 1.0 % agarose gel containing ethidium bromide in TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA) and visualisation under UV light. A 1 Kb Ladder (Gibco BRL, Life Technologies, Gaithersburg, MD, USA) was included on each gel to enable confirmation of the size of the amplified product.

1.2.1.4 Colony PCR

A 1 µL disposable loop was used to scrape colony material from the surface of a blood agar plate into 200 µL sterile phosphate buffered saline (PBS) in a PCR tube. After vortexing, the suspension was heated on the thermal cycler at 98 ^{o}C for 15 min. The cell debris was pelleted by centrifuging in a benchtop microfuge on full speed (13,000 rpm) for 5 min and 1 µl of the supernatant was used as template.

1.2.1.5 Universal 16S PCR

This PCR is capable of detecting all bacterial species. It was used in parallel with the species-specific PCR to verify specificity testing results (to prove that negative results were not false negatives.)

The 50 µl reaction mixture contained 1 mM $MgCl_2$, 50 mM KCl, 10 mM Tris HCl (pH 8.3), 100 µg/mL gelatin, 200 µM each dNTP (dATP, dCTP, dGTP, dTTP), 0.4 µM each primer (27F and 1525 R derived from Lane [1991]), the appropriate template and 0.8 units *Taq* DNA polymerase (Boehringer Mannheim).

The PCR was performed using a Hybaid OmniGene Thermal Cycler. An initial heating step at 98°C for 2.5 min was performed prior to the addition of the *Taq* DNA polymerase. A 50 µl mineral oil overlay was then added. The cycling parameters were: denaturing at 93°C for 1 min, annealing at 52°C for 45 s and extension at 72°C for 2 min, these three steps being repeated for 30 cycles. This was followed by a final cycle with an extension step of 10 min. PCR products were visualised as described above.

1.2.1.6 Sequencing

For 16S and 23S sequencing, the sequencing template was prepared by PCR amplification. PCR product for sequencing was purified using the QIAquick PCR Purification kit (QIAGEN GmbH, Hilden, Germany). DNA sequencing was performed using the ABI Prism™ Dye Terminator Cycle Sequencing Ready Reaction Kit with Amplitaq® DNA Polymerase FS and a model 373A DNA sequencing system (PE Applied Biosystems Inc., Foster City, CA, USA). All procedures were performed according to the manufacturer's protocols.

1.2.1.7 Cloning and sequencing

PCR products generated by *Pwo* DNA polymerase (Boehringer Mannheim) were cloned into the vector $pCAP^S$ using the PCR Cloning Kit (Blunt End) (Boehringer Mannheim). The vector was transformed into Epicurian Coli® XL1-Blue Competent Cells (Stratagene, La Jolla, CA, USA). Plasmid DNA was extracted from transformants using the High Pure Plasmid Isolation Kit (Boehringer Mannheim). Plasmid DNA was sequenced using the ABI Prism™ BigDye™ Terminator Cycle Sequencing Kit with Amplitaq® DNA Polymerase FS and a model 377 DNA sequencing system (PE Applied Biosystems Inc.). All procedures were performed according to the manufacturer's protocols.

1.2.2 Specific methods

1.2.2.1 16S ribosomal RNA approach

The first approach adopted in this study was to examine published sequence data for potentially suitable primers. 16S ribosomal RNA sequence data obtained by Dewhirst *et al.* (1992) was examined visually for regions where the sequence of *P. multocida* differed from that of other members of the family *Pasteurellaceae* found in chickens such as *P. gallinarum*, *P. avium*, *P. volantium* and *H. paragallinarum*.

Suitable primers were chosen and the PCR was optimised. Preliminary specificity testing was performed using DNA from the three subspecies of *P. multocida* as well as the 12 organisms in Table 1. The DNA samples were simultaneously amplified in the universal 16S PCR to demonstrate that false negatives were not occurring because of problems with the DNA.

Unexpected results with *P. multocida* subsp. *septica* necessitated sequencing of regions of the 16S gene of this strain, as published data was only available for subsp. *multocida*. *P. multocida* subsp. *gallicida* was included for completeness.

1.2.2.2 23S ribosomal RNA approach

As there was no published 23S ribosomal RNA sequence data for *P. multocida* it was necessary to generate original data during this study.

Using the primers identified by Van Camp *et al.* (1993) which target universally conserved regions flanking highly variable regions, sequence data for the three subspecies of *P. multocida*, as well as *P. gallinarum* and *H. paragallinarum* was generated.

Suitable primers were chosen and two PCR tests were optimised. Preliminary specificity testing was performed using DNA from the three subspecies of *P. multocida* as well as the 12 organisms in Table 1. The DNA samples were simultaneously amplified in the universal 16S PCR to demonstrate that false negatives were not occurring. One test was chosen for further evaluation, described in the next section.

1.3 Results

1.3.1 Results of 16S ribosomal RNA approach

Two primers were selected for testing:
the forward primer PM16F 5’ GAT GTT GTT AAA TAG ATA GCA TCA and
the reverse primer PM16R 5’ CAC AAG CTC ATC TCT GAG CTC.

The optimal $MgCl_2$ concentration was found to be 2 mM and the optimal primer concentration was 0.2 μM. The optimum annealing temperature was 60°C.

P. multocida subspecies *multocida* and *gallicida* were strongly amplified in this PCR. However, *P. multocida* subsp. *septica* was only weakly amplified. The 16S gene sequencing performed in this study indicated that there are seven nucleotide differences between subsp. *multocida* and subsp. *septica* in the region of the reverse primer of which four are concentrated at the 3’ end of the primer. This end is critical for successful extension. In other words, the sequence differences explained why subsp. *septica* was not amplified well in the 16S-based PCR. This was a critical flaw as it is important for any diagnostic test to be able to positively identify all three subspecies.

In addition, *P. canis* biovar 1 gave an amplified product in the 16S-based PCR. While this bacterium is not found in poultry, it was considered desirable to attempt to develop a more specific assay. Hence the study turned to the 23S ribosomal RNA.

1.3.2 Results of 23S ribosomal RNA approach

1.3.2.1 Preliminary work

From visual examination of the 23S ribosomal RNA sequence data generated for *P. multocida*, *P. gallinarum* and *H. paragallinarum*, three primers were designed. These were:
PM23F1: 5’ GGC TGG GAA GCC AAA TCA AAG
PM23R1: 5’ TCC CCT ACC CAA CAG AAT AAA TT
PM23R2: 5’ CGA GGG ACT ACA ATT ACT GTA A

A PCR test designated the PM 23-1 PCR, based on primers PM23F1 and PM23R1, was optimised with a $MgCl_2$ concentration of 4 mM, a primer concentration of 0.4 μM and an annealing temperature of 68°C.

Preliminary testing was done using DNA. All three subspecies of *P. multocida* amplified equally well. However, of the 12 members of the family *Pasteurellaceae* listed in Table 1, *P. canis* biovar 1 gave an amplified product.

A PCR test designated the PM 23-2 PCR, based on primers PM23F1 and PM23R2, was optimised with a $MgCl_2$ concentration of 3 mM, a primer concentration of 0.5 μM and an annealing temperature of 69°C.

Preliminary testing was done using DNA. All three subspecies of *P. multocida* amplified equally well in the PM 23-2 PCR. When the 12 members of the family *Pasteurellaceae* listed in Table 1 were tested in the PM 23-2 PCR and the 16S universal PCR, none were amplified in the PM 23-2 PCR, while as expected, all 12 were amplified in the universal PCR (Figure 1). This indicated that the test was capable of specifically detecting *P. multocida*, and further work was warranted.

Figure 1. Results of preliminary specificity testing of PM 23-2 PCR

Row A: amplified products from PM 23-2 PCR. Row B: amplified products from universal 16S PCR. Lane 1 is a molecular size marker. Lanes 2 to 16 are respectively *P. avium*, *P. volantium*, *P. gallinarum*, *P. anatis*, *P. dagmatis*, *P. langaa*, *P. stomatis*, *P. canis* biovar 1, *P.* spp A, *P.* spp B, *A. salpingitidis*, *H. paragallinarum*, *P. multocida* subsp. *multocida*, *P. multocida* subsp. *septica*, *P. multocida* subsp. *gallicida*. Lane 17 is a negative control. Only the three subspecies of *P. multocida* have been amplified in the PM 23-2 PCR while all templates have been amplified in the universal PCR.

Colony PCR

To facilitate the screening of large numbers of isolates, it was necessary to develop a colony PCR protocol. This obviates the need for DNA extraction, which is time-consuming. Initial attempts using a protocol developed for *H. paragallinarum* were unsuccessful. This protocol involved suspending cells in PBS and heating at 98°C for 5 min. Eventually, it was demonstrated that a heating time of 15 min was required to permit satisfactory amplification of the resultant template from *P. multocida* strains. Hence the method described in section 1.2.1.4 was adopted.

1.3.2.2 Use of colony PCR to determine universality of PM 23-2 PCR

Screening of reference strains of *P. multocida*

The PM 23-2 PCR had already been demonstrated to work well on the type strains of all three subspecies of *P. multocida*. Using the colony PCR protocol described above, the 16 reference strains for the Heddleston typing scheme and the two reference strains for the Carter typing scheme were shown to be positive in the PM 23-2 PCR.

Screening of avian isolates of *P. multocida*

Eighty-seven field isolates collected from poultry from the eastern States of Australia between 1966 and 1997 were included in the study. All 87 isolates were amplified in the PM 23-2 PCR.

The majority of these isolates gave a single amplified product of the expected size, 1.6 kb. However, a small number of isolates (16/87) gave unexpected products in the PM 23-2 PCR. There appeared to be four forms of this unusual product. Some isolates produced a single amplification product that was slightly longer than the standard product ("L" in Table 4). Other isolates produced two products, which appeared to consist of the standard product as well as the slightly longer product. This double product occurred in three forms. In some isolates the products were of equal intensity ("D,E" in Table 4), whereas for other isolates either the longer ("D,L") or the standard product ("D,S") was more intense. These differences were totally reproducible for each isolate.

Table 4. Details of isolates giving unusual PCR products in the PM 23-2 PCR

PM Code	Source	Date	Biotype	Cluster	ET	PCR
18	Chicken	2/86	3	A	28	D,E
36	Chicken	13/6/85	9	B	46	D,L
44	Chicken	26/9/85	9	B	45	D,L
77	Chicken	12/5/80	3	A	26	D,S
81	Poultry		6	B	52	D,E
89	Chicken	7/86	9			L
90	Chicken	10/8/88	9	B	46	D,L
91	Chicken	10/86	2	B	48	D,S
131	Poultry		2	C	56	D,E
135	Turkey	12/92	10	B	43	L
140	Poultry		7	B	44	D,E
141	Poultry		1	B	42	L
142	Poultry		2			D,E
146	Poultry		5			D,L
148	Poultry		5			D,L
227	Turkey	29/09/94				D,S

Cloning and sequencing of unusual PCR products

Cloning and sequencing of PM 89 (a representative "L" pattern) revealed that there was a 126 bp insert in the PCR product that was not present in the standard PCR product given by the type strain NCTC 10322.

Screening of porcine isolates of *P. multocida*

As the laboratory also had a large number of porcine isolates of *P. multocida* in the culture collection, 36 well-characterised isolates were tested in the PM 23-2 colony PCR. All were positive and all produced a single 1.6 kb amplified product.

1.3.2.3 Further testing of the specificity of the PM 23-2 PCR

Type strains

To further investigate the specificity of the PM 23-2 PCR, a number of other members of the family *Pasteurellaceae* (listed in Table 2) were tested in this PCR in parallel with the

universal PCR. Some of these were chosen to represent bacteria likely to be present in pigs, to determine whether the PCR might also be useful for the diagnosis of porcine pasteurellosis. Additional type strains representing bacteria commonly found in poultry (listed in Table 3) were also included.

Several studies have shown that there are three *Pasteurella* species associated with calf pneumonia: *P. multocida* subsp. *multocida*, *P. canis* biovar 2 (also referred to as Orn^+ Bisgaard Taxon 13) and *P. avium* biovar 2 (also referred to as Orn^- Bisgaard Taxon 13) (Madsen *et al.* 1985; Abdullahi *et al.* 1989; Bisgaard *et al.* 1991). By "simplified routine bacteriological examinations" all three are commonly identified as *P. multocida* (Bisgaard *et al.* 1991). The two species formerly known as Taxon 13 were included in the present study because of their close relationship to *P. multocida*, although they are not found in avian hosts.

The results are shown in Table 5.

Table 5. PM 23-2 PCR results on additional type strains

Organism	Code Number	PM 23-2 PCR result
Actinobacillus pleuropneumoniae	ATCC 27088	-
Actinobacillus pleuropneumoniae	HIM 677-34	-
Actinobacillus suis	CCUG 11624	-
Haemophilus Taxon C	CAPM 5111	-
Pasteurella avium biovar 2	CCUG 16497	+
Pasteurella canis biovar 2	HIM 843-5	+
Pasteurella haemolytica	CCUG 408	-
Pasteurella trehalosi	CCUG 27190	-
Alcaligenes faecalis	ATCC 8750	-
Bordetella avium	ATCC 35086	-
Bordetella bronchiseptica	ATCC 19395	-
Bordetella hinzii	LMG 13501	-
Ornithobacterium rhinotracheale	CCUG 23171	-

Field strains

Multiple isolates of a range of predominantly poultry organisms such as *A. salpingitidis*, *H. paragallinarum*, *P. gallinarum, B. avium*, and *B. hinzii*, were tested in both the PM 23-2 PCR and the universal PCR. Single field isolates of avian *E. coli*, *S. aureus*, and *S. hyicus* were also included. Additionally a few porcine and one bovine organism as listed in section 1.2.1.1 were tested. All were negative in the PM 23-2 PCR and positive in the universal PCR.

1.4 Discussion

The objective of this part of the project was to develop a rapid and accurate molecular method for the identification of *P. multocida*, the causative agent of fowl cholera. The PM 23-2 PCR test developed in this project meets this objective.

The PM 23-2 PCR test has been shown to be capable of detecting all three subspecies: *P. multocida* subsp. *multocida*, *P. multocida* subsp. *gallicida* and *P. multocida* subsp. *septica*. While *P. multocida* subsp. *multocida* is by far the most commonly isolated subspecies from Australian poultry (Fegan *et al.* 1995), it is important for any diagnostic test to be capable of detecting all three subspecies. The PM 23-2 PCR meets this requirement.

The PM 23-2 PCR test also correctly identified 18 overseas serovar reference strains, indicating that it would be capable of detecting a wide variety of *P. multocida* strains. The robustness of the PCR is indicated by the fact that these overseas reference strains have been collected from at least seven different animal hosts. The applicability of the PM 23-2 PCR was further demonstrated by the correct identification of 87 Australian avian field isolates, representing a collection with geographic, temporal and genetic diversity. Among this collection were two field isolates of subsp. *septica* and three of subsp. *gallicida.*

In addition, 36 porcine isolates were positive, suggesting that the PCR might be applicable to the diagnosis of porcine pasteurellosis. Worldwide, porcine pasteurellosis remains one of the most important diseases associated with intensive pig production. Hence the PM 23-2 PCR should be of considerable benefit to the pig industry (both in Australia and overseas) as well as to the diagnostic laboratories that serve the pig industry.

Of the 24 type strains of other organisms tested all were negative except for *P. avium* biovar 2 and *P. canis* biovar 2. None of the six additional organisms tested as field strains but not as type strains were positive in the PM 23-2 PCR. These organisms were predominantly of avian or porcine origin.

P. avium biovar 2 and *P. canis* biovar 2 were included in the present study for reasons of completeness. Despite its name, *P. avium* biovar 2 has only ever been isolated from calves and the same is true for *P. canis* biovar 2. However, Dewhirst *et al.* (1993) had demonstrated that by 16S ribosomal RNA analysis *P. avium* biovar 2 was very closely related to *P. multocida*. Given that the PM 23-2 PCR is based on the 23S ribosomal gene, it seemed likely that it might amplify both of these organisms. Thus the PCR result was not unexpected. The taxonomy of this group has been in a state of flux for the past fifteen years and continuing study may well result in the reclassification of these biovar 2 variants. As neither *P. avium* biovar 2 nor *P. canis* biovar 2 occur in poultry or pigs, it is true to say that *P. multocida* is the only organism of avian or porcine origin shown to give a positive reaction in the PM 23-2 PCR.

In summary, a very comprehensive range of avian and porcine bacteria all gave negative results in the PM 23-2 PCR. For this reason the PM 23-2 PCR can be used to confidently identify *P. multocida* in specimens originating from poultry or pigs.

The conventional method for the identification of a suspect isolate as *P. multocida* requires that the isolate be subjected to a range of biochemical tests. This range of biochemical tests has to be extensive as a number of close relatives of *P. multocida* occur in poultry. Specifically, the phenotypic testing must be complete enough to separate the following members of the genus *Pasteurella,* all of which are recognised as being present in avian hosts: *P. anatipestifer* (also known as *Riemerella anatipestifer), P. anatis, P. avium, P. gallinarum, P. haemolytica* (also known as *Actinobacillus salpingitidis), P. langaa, P. multocida* (existing in three subspecies), *P. volantium* and *Pasteurella* species A. The reality is that the task of confidently identifying a suspect *P. multocida* by the conventional, phenotypic methods

requires an extensive range of testing that is beyond the capability of routine diagnostic laboratories.

The PCR test has significant advantages over this conventional approach. The PM 23-2 PCR enables very rapid confirmation of a suspect colony. The PCR test can give results in less than five hours, compared with two to five days for the conventional approach. As well, the identification given by the PM 23-2 PCR has been shown in this project to be very specific.

One puzzling issue raised by this study relates to the difference in PCR products generated from less than 20% of the field strains tested. This only occurred among avian strains. There may be some biological significance in this finding in that most of these isolates fall into a small subgrouping of avian *P. multocida* termed cluster B by Blackall *et al*. (1996). This cluster B was recognised by the use of multi-enzyme electrophoresis and was supported by the use of ribotyping. The cluster B isolates of *P. multocida* are a diverse group of biochemically unusual organisms. The unusual nature of cluster B has been further underlined by the fact that most (but not all) of the isolates giving unusual PCR products in the PM 23S-2 PCR were located in cluster B.

During the course of this project, another PCR test for *P. multocida* was published (Kasten *et al.* 1997). This PCR test is based on a gene (*psl*) that encodes a protein that is found only in *P. multocida* and *Haemophilus influenzae*. The latter organism is not normally isolated from poultry. The specificity of the test was examined using DNA from a range of avian and porcine bacteria. No false-positives were obtained, although as expected, *H. influenzae* was amplified. Unfortunately *P. avium* biovar 2 and *P. canis* biovar 2 were not included in this study.

There are also several papers reporting the use of PCR to detect toxigenic *P. multocida* associated with atrophic rhinitis in pigs (*e.g.* Kamp *et al.* 1996). Of course these tests are not relevant to poultry as they recognise the presence of the toxin gene which is not present in avian isolates of *P. multocida.*

In summary, the PM 23-2 PCR represents a considerable step forward in our ability to diagnose fowl cholera. The PCR is specific and rapid. The equipment and expertise required to perform PCR-based tests are now widely available in the central government laboratories that serve the Australian poultry industries. Hence, this assay should be capable of being made rapidly available to the Australian poultry industries.

Objective 2
Validation of two PCR tests for *Haemophilus paragallinarum*

2.1 Introduction

In this study, two PCR tests for *H. paragallinarum* (the HP-2 PCR and the 23S HP-PCR), that had been developed by this laboratory during an ACIAR-funded project, were validated in a number of trials on live chickens. Preliminary studies had indicated that the 23S HP-PCR was the more sensitive test, but it gave occasional false positive reactions with two out of six strains of *Pasteurella volantium i.e.* it lacked absolute specificity.

The experiments described below were designed to answer two main questions:
1. which of the two PCR tests should be recommended for use in suspected field outbreaks of infectious coryza?
2. how should swabs be transported from the field to the laboratory?

Previous validation work on the HP-2 PCR had focussed on samples obtained at necropsy from chickens that had been artificially infected with *H. paragallinarum*. Two further artificial challenge trials were conducted on live birds using the 23S HP-PCR. Then, as there were few outbreaks of infectious coryza in SE Queensland during the course of the project, two pen trial experiments were designed to imitate the spread of infectious coryza through a flock under field conditions. Subsequently the PCR tests were used in three real outbreaks.

2.2 Methodology

2.2.1 PCR protocols

The first of the two PCR tests, termed the HP-2 PCR, was developed by Chen *et al.* (1996). This test is based on a unique cloned fragment of DNA that was found to be specific for *H. paragallinarum*.

For the HP-2 PCR, the 50 µl reaction volume consisted of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 0.01% (wt:vol) gelatin, 200 µM each of dATP, dGTP, dTTP and dCTP, 0.4 µM of each primer, the appropriate amount of template and 1.25 U of *Taq* DNA polymerase (Boehringer Mannheim). Amplification was performed using a Hybaid OmniGene thermal cycler in which samples were denatured at 98°C for 2.5 min before *Taq* and mineral oil were added. The amplification conditions consisted of 25 cycles of 94°C for 1 min, 65°C for 1 min and 72°C for 2 min followed by a final cycle of 94°C for 1 min, 65°C for 1 min and 72°C for 10 min.

The second PCR test, termed the 23S HP-PCR, uses primers derived from the 23S ribosomal RNA sequence of *H. paragallinarum* (Miflin, unpublished data).

For the 23S HP-PCR, the 50 µl reaction volume consisted of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1 mM $MgCl_2$, 0.01% (wt:vol) gelatin, 200 µM each of dATP, dGTP, dTTP and dCTP, 0.4 µM of each primer, the appropriate amount of template and 1.25 U of *Taq* DNA

polymerase. The amplification conditions consisted of 30 cycles of 94°C for 1 min, 68°C for 1 min and 72°C for 1 min followed by a final cycle of 94°C for 1 min, 68°C for 1 min and 72°C for 10 min.

Each PCR test was run simultaneously on a separate block of the Hybaid Omnigene thermal cycler. A positive control consisting of *H. paragallinarum* DNA and a negative control containing no DNA were included in each run on each machine. PCR products were detected by horizontal gel electrophoresis of a 10 µl aliquot in a 1.0 % agarose gel containing ethidium bromide in TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA) followed by visualisation under UV light. A 1 Kb Ladder (Gibco BRL) was included on each gel to enable confirmation of the size of the amplified product.

2.2.2 Colony PCR method

For confirmation of the identity of typical satellitic colonies on blood agar, colony PCR was performed. The PCR reaction mixes and cycling parameters were as described above. The template was prepared by suspending some colony material from an agar plate into 200 µl phosphate buffered saline (PBS). After heating at 98°C for five min the cells were pelleted by centrifuging in a bench-top microfuge for 5 min at 13000 rpm. One µl of the supernatant was used in the PCR test.

2.2.3 General protocols for validation trials

2.2.3.1 Live bird sampling procedure

Each bird was first inspected visually for clinical signs of infectious coryza (facial swelling, nasal discharge). The bird was then held while gentle pressure was exerted on each infra-orbital sinus. If mucus was expressed, it was sampled using a 1 µl sterile disposable loop for inoculation onto blood agar plates for conventional culture. The mucus was also collected onto a sterile cotton-tipped swab for PCR testing.

2.2.3.2 Sample processing- culture

After inoculation with mucus from the loop, blood agar plates were cross-streaked with a culture of *Staphylococcus hyicus* and incubated for 24 and 48 h at 37°C in an atmosphere of 5% CO_2.

2.2.3.3 Sample processing- PCR

The swab was soaked in PBS for 1 h at room temperature. Using forceps, the swab was squeezed out and discarded. The suspension of mucus in PBS was vortexed briefly then centrifuged at 13000 rpm for 15 min. The supernatant was discarded and the pellet re-suspended in 20 µl PBS. Five µl of this suspension was transferred to 45 µl lysis buffer (10 mM Tris-HCl pH 8.3, 200 mg/ml proteinase K, 0.5% Tween 20, 0.5% Nonidet P 40) and incubated at 56°C for 1 h to lyse the cells and release DNA. To inactivate the proteinase K, which would interfere with the PCR, the samples were heated at 95°C for 10 min. Twenty µl was used as template in each PCR test.

2.2.3.4 Statistical analysis

Differences between the various treatment groups in the experiments described in this report were examined using Fisher's exact test (two tailed test, 95% confidence level) (GraphPad Prism Software, GraphPad Software Inc).

2.2.4 Artificial challenge trial protocols

Two preliminary trials were conducted to validate the use of the 23S HP-PCR on live chickens that had been inoculated with *H. paragallinarum*.

2.2.4.1 Trial 1

Trial 1 consisted of 30 Ross chickens. All 30 chickens were artificially infected by intra-sinus inoculation with 4.3×10^7 cfu of an overnight broth culture of *H. paragallinarum* strain HP14, an Australian field strain. All chickens were examined by the live bird sampling procedure described above at Days 2 and 7 post challenge. At Day 2, samples were taken from both sinuses if available. At Day 7, samples were taken from one sinus only. The mucus samples were examined on the day of collection by the 23S HP-PCR and culture.

2.2.4.2 Trial 2

Trial 2 consisted of 20 SIRO CB layers. All 20 chickens were artificially infected as described above with 4.2×10^7 cfu of *H. paragallinarum* strain HP14. All chickens were examined at Days 2, 5 and 7 post challenge and mucus samples were taken from both sinuses if available. The samples were tested as in Trial 1. Samples that tested negative were retested from the resuspended swab material.

The presence of inhibitory substances was investigated by the addition of 2 µl of swab suspension into a PCR reaction mix containing amplifiable positive control DNA.

2.2.5 Natural challenge trial protocols

2.2.5.1 Trial 3

This trial utilised 20 specific-pathogen-free (SPF) chickens from the Animal Research Institute flock. Five birds selected at random were inoculated with 2.8×10^7 cfu of *H. paragallinarum* HP14 as described above. These birds were allowed to run free with the 15 uninoculated chickens, which were thus exposed to a natural challenge of infectious coryza. All chickens were checked at Days 4, 7, 9, 11, 14, 17 and 22 post challenge. Mucus samples were then examined by both the 23S HP-PCR and the HP-2 PCR and by culture.

2.2.5.2 Trial 4

A similar trial was conducted using 24 commercial SIRO CB pullets, purchased three weeks prior to point of lay. The birds were not vaccinated against infectious coryza. Five birds selected at random were inoculated with 1.3×10^7 cfu of *H. paragallinarum* HP14. These birds were allowed to run free with the 19 uninoculated chickens. On each sampling occasion (Day 2, 4, 7, 9, 11, 15 and 25 after inoculation), each bird was inspected visually and mucus

samples collected as described above. Where sufficient mucus was available, extra samples were taken for simulated transport experiments (see below).

In this trial, one set of samples was collected and examined on the same day. In addition, experiments were set up to mimic transport of samples from the field to a central testing laboratory. The simulated transport conditions consisted of holding the swabs, either in phosphate buffered saline (PBS) with 30% glycerol or as dry swabs, in an esky with chilly bricks for either 24 or 72 hours. After storage, the swabs were streaked onto blood agar plates, cross-streaked and incubated as above and processed for testing in both PCRs.

To evaluate the sensitivity of the PCR tests, certain swabs were chosen for further evaluation. Swab suspensions were serially diluted and aliquots taken into lysis buffer and for enumeration on TM/SN agar (Reid and Blackall 1987) (a specially supplemented medium that supports the growth of *H. paragallinarum*).

2.2.6 Field outbreak situations

2.2.6.1 Outbreak 1

The first outbreak occurred in a commercial egg farm near Brisbane. Birds were suffering from extremely severe clinical signs: “tennis ball” heads. The birds were subsequently diagnosed as suffering from Marek’s disease. Mucus samples were taken from the sinuses of 15 live birds without gross clinical signs that were sharing cages with severely affected birds. An additional two birds were examined after post-mortem. All samples were examined by both PCR tests and culture as described above on the day of sample collection.

2.2.6.2 Outbreak 2

The second suspected outbreak occurred in broiler breeder birds. Two birds were submitted for examination. These birds were live bird sampled by the method described above. As well, at the suggestion of the submitting veterinarian, swabs were taken from the palatine cleft. This site was chosen because it is the place where samples are taken for the diagnosis of respiratory *Mycoplasma* infection. Samples were also collected from each sinus after necropsy. All samples were processed on the day of collection.

2.2.6.3 Outbreak 3

The third suspected outbreak occurred in 86-week old commercial layers in NSW. Three palatine cleft swabs stored in Amies medium with charcoal were received at the laboratory nine days after collection. These swabs were processed on day of receipt as described in sections 2.2.3.2 and 2.2.3.3.

2.3 Results

2.3.1 Trial 1

This trial involved 30 Ross chickens, artificially challenged with *H. paragallinarum*.
Table 6. Results of Trial 1

Day	# samples	# samples positive in culture	positive in 23S HP-PCR
2	31	30[a]	30[a]
7	20	16[a]	13[a]

Within a row, values that share a superscript are not significantly different ($p \leq 0.05$).

Statistical analysis showed no significant difference between culture and the 23S HP-PCR at Day 2 or Day 7.

2.3.2 Trial 2

This trial involved 20 SIRO CB layers artificially challenged with *H. paragallinarum*.

Table 7. Results of Trial 2

Day	# samples	# samples positive in culture	positive in 23S HP-PCR
2	40	40[a]	35[a] (39)[a] *
5	31	31[a]	26[a] (31)[a]
7	30	30[a]	26[a] (28)[a]

*The numbers in brackets include the swabs which were initially negative but positive on retest.
Within a row, values that share a superscript are not significantly different ($p \leq 0.05$).

Statistical analysis showed no significant difference between culture and the 23S HP-PCR at Day 2, 5 or 7. No significant difference was detected between culture and PCR irrespective of whether or not the retested samples were included in the analysis.

Of the four samples from Day 2 that originally tested PCR negative but were positive on retest, only one contained blood. This raised the possibility that there might be inhibitory substances other than blood present in some mucus samples. This was demonstrated for the sample from Day 2 that remained negative on retest. Two µl of this swab suspension completely inhibited the amplification of the positive control DNA. The nature of this inhibitory substance is unknown.

2.3.3 Trial 3

This natural challenge trial was conducted in SPF chickens. All five artificially infected chickens were positive by Day 4 of the trial and four of these were still positive at Day 17. Of the 15 uninoculated birds, eight remained uninfected throughout the trial.

Table 8 shows the number of positives for the two PCR methods and culture.

Table 8. Results of Trial 3

chickens positive by

Culture	HP-2 PCR	23S HP-PCR
90/105[a]	57/74[a]	71/91[a]

Within a row, values that share a superscript are not significantly different ($p \leq 0.05$).

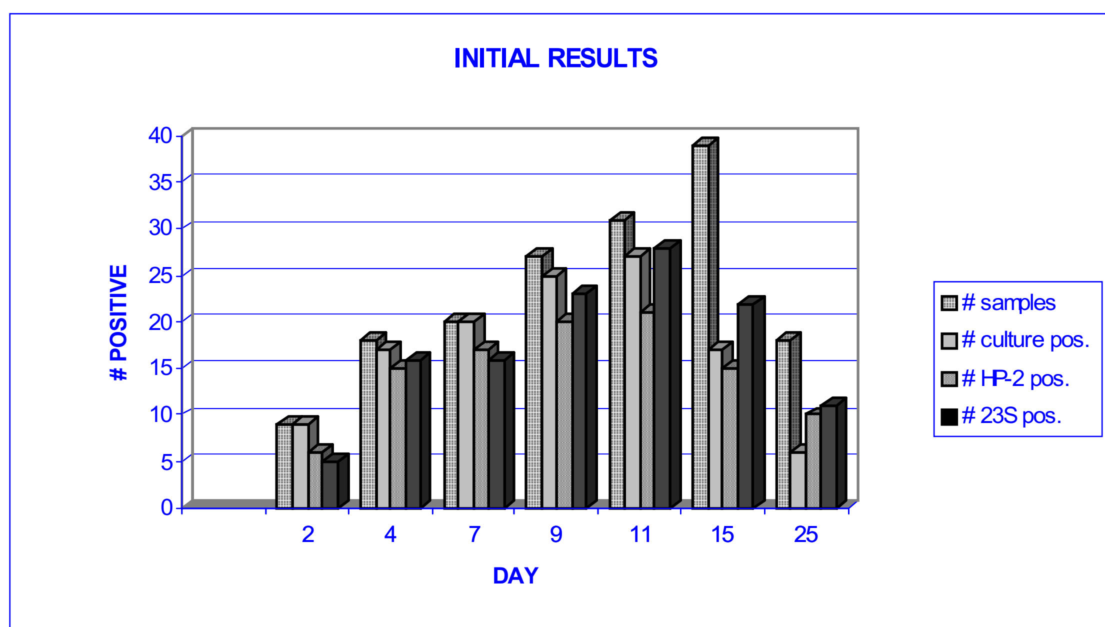
Statistical analysis revealed that there was no significant difference in any of the methods.

2.3.4 Trial 4

This natural challenge trial was conducted in commercial SIRO CB birds. All five artificially infected chickens were positive by Day 2 of the trial. Of the 19 uninoculated birds, only four appeared to remain uninfected throughout the 25 days of the trial.

The results for each sampling day are illustrated in Figure 2. Of 162 samples collected and tested on the same day, 121 (75%) were positive by culture and the same number were positive in the 23S HP-PCR. Only 104 (64%) were positive in the HP-2 PCR. Although the difference between the two PCR tests was not statistically significant (p = 0.0534), there is a clear trend towards the conclusion that the 23S HP-PCR was equivalent to culture and superior to the HP-2 PCR.

Figure 2. Results of Trial 4: samples tested on day of collection



The results obtained using samples tested after storage are set out in Table 9. The results are presented as the number of samples that were positive in each test.

Table 9. Results of Trial 4 storage study

Time	PBS + 30% glycerol			DRY		
	Culture	HP-2 PCR	23S HP-PCR	Culture	HP-2 PCR	23S HP-PCR

24 hours (28 samples)	23[a,b]	25[a,b]	25[a,b]	20[a]	26[a,b]	28[b]
72 hours (45 samples)	5[a]	31[b]	35[b]	0[a]	38[b]	39[b]

Within a row, values that do not share a superscript are significantly different ($p \leq 0.05$).

Statistical analysis showed no significant difference between the two PCR tests after either 24 hours or 72 hours storage, regardless of whether the swabs were stored in PBS/glycerol or dry. However, there were some significant differences between results given by culture and PCR. After 24 hours storage of the dry swabs, the 23S HP-PCR, but not the HP-2 PCR, gave significantly more positive results than culture. After 72 hours storage, both PCR tests (on swabs stored in either PBS/glycerol or dry) gave significantly more positives than culture (on swabs stored in either PBS/glycerol or dry).

In comparing the sensitivity of the two PCR tests using sinus swabs as the source material, the 23S HP-PCR was able to detect at least 10 times fewer organisms than the HP-2 PCR. In one sample the difference was 1000 fold. The minimum detection limit of the 23S HP-PCR was 30 colony forming units.

2.3.5 Outbreak 1

The 15 live birds from the commercial egg farm generated 16 samples, as one bird was sampled from both sinuses. Of these 16 samples, seven (44%) were positive in the HP-2 PCR and 13 (81%) were positive in the 23S HP-PCR. The two birds examined after post-mortem generated a total of four samples of which one was positive in each PCR test.

It was impossible to identify satellitic colonies typical of *H. paragallinarum* on the culture plates, meaning that classic, traditional cultural confirmation of *H. paragallinarum* could not be performed. Instead, the two PCR tests were performed on suspect colony groups. Of the 13 picked for testing, seven were confirmed to contain *H. paragallinarum* by both PCR tests. In this way, five of the 15 live birds and one of the post-mortem birds were confirmed positive by this combination of culture followed by PCR confirmation of colony sweeps.

2.3.6 Outbreak 2

In the second disease investigation, two chickens from a broiler breeder flock were submitted for testing by culture and both PCR tests. Of the 10 samples tested from these two birds, three were positive in the 23S HP-PCR. These were a left sinus from one bird (live-bird sample) and the palatine cleft from both birds. No samples were positive in the HP-2 PCR. No typical *H. paragallinarum* colonies were confirmed from any of the culture plates.

As no actual isolates of the organism responsible for the positive result in the palatine cleft of these broiler breeder birds were obtained, the only available technique for further investigation was sequencing of the PCR products generated by the 23S HP-PCR. The sequence of the PCR product amplified in the 23S HP-PCR from the palatine cleft swab taken from one of these birds was compared with that for five isolates of *H. paragallinarum* and the two *P. volantium* isolates that are amplified in this PCR. Unfortunately, any nucleotide differences that were identified were scattered so that no definitive answer could be obtained as to the identity of the palatine cleft organism.

In order to obtain more information about the palatine cleft as a site for sampling for the diagnosis of infectious coryza, palatine cleft swabs were taken from the 24 birds that had been used in Trial 4. Of the 24 samples taken 29 days after challenge (*i.e.* 4 days after the trial was completed), five were positive in the HP-2 PCR and all 24 were positive in the 23S HP-PCR. Again, it was very difficult to find colonies on the plates that resembled *H. paragallinarum*. Of six colony sweeps selected for confirmatory testing by PCR, all were positive in the 23S HP-PCR but only one was positive in the HP-2 PCR.

Palatine cleft swabs were also obtained from 12 ten-week old birds from the ARI SPF flock (a flock known to be free of *H. paragallinarum*). All 12 samples were negative in both PCRs.

2.3.7 Outbreak 3

Despite the fact that the three swabs obtained from the layers in this suspected outbreak were submitted in a completely different transport medium that had not been previously validated, and that they were received nine days after collection, all three were positive in both PCR tests. Culture plates inoculated from the nine day old swabs were totally overgrown, an expected result given the length of time between collection and testing.

2.4 Discussion

Until the recent work on a PCR test for *H. paragallinarum* performed at this laboratory, the traditional means of confirming infectious coryza in a chicken flock remained the isolation and biochemical characterisation of *H. paragallinarum* (Blackall *et al.* 1997). This is a technically demanding task as isolates of *H. paragallinarum* are nutritionally demanding, requiring special supplements to allow them to grow in artificial media. Further, *H. paragallinarum* is a slow growing organism that is easily overgrown by other bacteria. Even if a suspect isolate is obtained, the diagnostic laboratory must still perform tests to distinguish *H. paragallinarum* from similar, but non-pathogenic, bacteria.

Recently, the diagnostic options for infectious coryza have been expanded by the availability of a species-specific PCR test for the identification of *H. paragallinarum* (Chen *et al.* 1996). This test, termed the HP-2 PCR, was developed and validated at this laboratory in artificially infected chickens in small scale pen trials (Chen *et al.* 1996). We have recently developed a second test - the 23S HP-PCR (Miflin, unpublished data). The aim of the current study was to directly compare these two PCR tests under realistic conditions typical of those facing diagnostic laboratories investigating suspected coryza outbreaks in commercial chickens.

In reviewing the results of this study it is important to understand that the work was performed at a laboratory that has extensive experience in the traditional culture methods for the isolation of *H. paragallinarum*. The Bacteriology Research Laboratory has been working on the traditional diagnosis of infectious coryza since 1979 and has vastly more experience in this difficult technique than the typical diagnostic laboratory. Hence, it is highly likely that the culture results obtained in this study are better than would be obtained in a diagnostic laboratory that has not had the opportunity of developing such extensive experience.

As the 23S HP-PCR has only recently been developed, we initially performed two artificial challenge trials (Trials 1 and 2) to allow us to evaluate the test when used directly on infected

chickens. These trials demonstrated that the 23S HP-PCR gave equivalent results to culture when used on mucus samples taken from live birds. It should be stated at the outset that one of the drawbacks encountered when validating the live bird sampling technique was that negative samples could not be tested. A healthy chicken does not produce mucus and so does not generate a sample for testing. Another factor to be considered is that culture is relatively easy to interpret when samples have been taken aseptically with a tiny loop from artificially challenged birds. These birds have high numbers of *H. paragallinarum* present in the mucus in relatively pure culture. This is unlikely to be the case in a real outbreak.

Clearly, both the 23S HP-PCR and the HP-2 PCR needed to be validated using samples from real outbreaks of infectious coryza. To simulate such a situation, two pen trials were conducted to imitate the natural history of the disease (Trials 3 and 4). The only difference between these trials was that Trial 3 was performed in SPF chickens while Trial 4 was performed in commercial chickens. No statistically significant difference was found between either PCR test and culture in either trial, provided the samples were tested on the same day. Hence, these two trials have, like the trials involving artificial infection, confirmed that the 23S HP-PCR and the HP-2 PCR are the equivalent of culture.

Trial 4 demonstrated the impact of storage/transport on both PCRs and traditional culture. After 72 hours of simulated transport in a chilled esky, there were significantly more positive results in both PCR tests than in culture, demonstrating that PCR is a more robust technique than culture. This is an important finding as many diagnostic laboratories are likely to be attempting to diagnose infectious coryza by culturing swabs transported to a central laboratory. Our work clearly shows that culture can only be a reliable technique when used on freshly obtained swabs.

The four pen trials demonstrated the suitability of live bird sampling as a means of obtaining mucus for both culture and PCR analysis. While the overall results suggest that both PCR and culture give equivalent results, the advantage of PCR is its rapidity. PCR results are available within six to eight hours while confirmed culture requires three to five days.

The natural outbreaks presented significant problems typical of those seen in routine diagnostic situations. For instance, in Outbreak 1 the birds were suffering from an immunosuppressive disease which meant that the cultures were severely contaminated with opportunistic organisms. Whereas in all the pen trials it was relatively easy to recognise *H. paragallinarum* colonies, in the field situation it was much more difficult. This difference is not statistically quantifiable but it is nevertheless very real. The difficulty of working with actual outbreaks in commercial chickens is emphasised by the fact that we were unable to obtain a single pure culture of *H. paragallinarum* from two of the three outbreaks of infectious coryza investigated. This was despite that fact that, in all three cases, the typical clinical signs of infectious coryza were present and the culture was performed in a very experienced laboratory. Indeed, it was only the PCR techniques, applied either directly to the living bird or to highly overgrown plate cultures, that allowed a diagnosis of infectious coryza to be made. If the three field investigations had been dependent upon traditional culture, none of the outbreaks would have been confirmed as coryza, despite the presence of the typical clinical signs and the field veterinarian's provisional diagnosis of coryza.

In Outbreak 3, all three swabs submitted were positive in both PCR tests, even though they were tested nine days after collection. This effectively demonstrates the power of PCR as the submitting laboratory was unable to isolate *H. paragallinarum* one day after swab collection.

In summary, this study has confirmed the suitability of the PCR tests for the diagnosis of outbreaks of infectious coryza in commercial poultry. In Outbreak 1, involving a commercial egg farm, the PCR tests were able to confirm the disease as infectious coryza on the same day the samples reached the laboratory. Culture required the use of PCR for confirmation as the plates were seriously overgrown. In the case of Outbreaks 2 and 3, only the PCR tests were able to detect positive chickens, with culture failing altogether.

The field work also revealed the possibility that the palatine cleft may be a good site for obtaining samples from living birds. The standard live bird sampling method requires the presence of sufficient mucus in the sinus to allow the mucus to be expressed by gentle pressure. This means that the sampling can only be done at limited stage of the disease process - when mucus is present in the sinus. Hence, live bird sampling of the mucus cannot be performed in the early stages of the disease, the later stages of the disease or when a carrier state has been reached. In contrast, it is possible to obtain a sample by swabbing the palatine cleft at all stages of the disease process. Further work would be required to validate the palatine cleft approach, involving more sampling of both positive and negative birds. The validation of palatine cleft sampling is made difficult by the fact that the site is heavily colonised by commensal bacteria, making it virtually impossible to culture *H. paragallinarum*, which is easily overgrown. Hence, the nature of the site would make a comparison of PCR and culture very difficult.

The aims of Objective 2 were to address two questions regarding the use of PCR in the diagnosis of suspected outbreaks of infectious coryza in Australia. The first question concerned a recommendation about which of the two PCR tests would be most suitable for use. In pen trials there was no statistically significant difference between the two PCR tests when used on mucus samples taken from live birds. However, quantitative analysis proved that the 23S HP-PCR could detect ten times fewer cells of *H. paragallinarum* in mucus than the HP-2 PCR. This finding suggests that in some field cases, the higher sensitivity of the 23S HP-PCR would theoretically mean that this test would perform better than the HP-2 PCR. This may explain the trend towards higher numbers of positives with the 23S HP-PCR as seen in Trial 4 as well as some of the results from the field outbreaks. However, the 23S HP-PCR does suffer from a lack of specificity. The test has given false positive reactions with two out of six strains of *P. volantium* tested. A series of attempts to solve this problem by increasing the stringency of the assay were unsuccessful, resulting only in the test failing to recognise some *H. paragallinarum* isolates. A better approach to this issue would be to design a second level assay, to be performed after a positive had been obtained in the 23S HP-PCR, that would distinguish between a positive due to *H. paragallinarum* and a positive due to *P. volantium*. Such a confirmatory test would need to be developed before the 23S HP-PCR could be recommended for use. However, at the present time, the HP-2 PCR can be confidently recommended as an alternative to, or replacement for, culture.

The second question related to transport of swabs from the field to the laboratory. Not surprisingly given the delicate nature of the organism (Blackall *et al.* 1997), the study demonstrated that the quicker the samples reach the laboratory, the greater the chance of detecting *H. paragallinarum*. However, Outbreak 3 demonstrated that it is possible to obtain results by PCR even after nine days. As there was essentially no difference in the performance of the two transport regimes examined in this study, our recommendation would be to transport mucus swabs dry and chilled, as this is the simplest procedure. It appears that

Amies medium with charcoal may also be suitable although it has not been extensively validated.

Implications

The diagnostic assays developed and/or validated in this project represent a major step forward in the capacity to rapidly and accurately diagnose both fowl cholera and infectious coryza.

Fowl cholera continues to be an important disease in both economic terms and prevalence throughout the Australian poultry industries (chicken meat and egg). Hence, the improved diagnostic capability resulting from the use of the PCR test will allow the adoption of improved prevention and control programmes, resulting in improved financial returns to the Australian industries.

Based on the traditional diagnostic assays, infectious coryza has been widely regarded as a relatively minor disease in terms of both incidence and economic impact in Australia. The results of this project have highlighted that the traditional method of culture is a very poor way of detecting the presence of *H. paragallinarum*, the causative agent of infectious coryza. The adoption of a PCR test which has been shown to be superior to culture in a field diagnostic situation, will result in a much improved diagnosis of infectious coryza. A rapid and specific diagnostic service based on this test will ensure that more effective prevention and control programmes for infectious coryza can be developed. It is also possible that the improved ability to diagnose infectious coryza will reveal a greater level of infection with *H. paragallinarum* than has previously been realised.

As PCR-based technology is now a feature in the central laboratories of all the State Departments of Agriculture/Primary Industries, the assays developed in this project are capable of being immediately provided in each State. The industry-based laboratories are generally not yet equipped for PCR, meaning that the poultry industries will have to access these assays from government laboratories in the short term. However, as PCR-based technologies are increasingly replacing traditional technologies, it is likely that the industry-based laboratories will also adopt PCR technology in the not too distant future. In addition, it is likely that the private laboratories servicing the diagnostic needs of the poultry industries will both increase in number and also use PCR-based technologies, providing yet another means of access to the tests developed in this project.

Recommendations

To ensure that the Australian poultry industries gain maximum benefit from the tests developed in this project, the PCR tests for *Pasteurella multocida* and *Haemophilus paragallinarum* should be recommended as either replacements for traditional culture-based methods or as supporting tests to be used in conjunction with culture. The Bacteriology Research Laboratory, which acts as an unofficial reference laboratory for fowl cholera and infectious coryza, has already adopted the tests for routine use. The change has been made because the PCR tests have been thoroughly validated, provide more rapid results than traditional methods and are cost-effective.

To ensure that the other research and diagnostic laboratories that service the Australian poultry industries are aware of the advantages of the PCR tests, the results of this project should be widely disseminated. In the spirit of this recommendation, aspects of this work have already been presented as oral papers at scientific meetings attended by diagnostic personnel that service the Australian poultry industries (*e.g.* Australian Veterinary Poultry Association).

The work is also being prepared for publication in the international scientific literature, ensuring that all diagnostic laboratories in Australia (and overseas) are aware of the capacities and features of the tests developed in this project.

A more extended application of the *Pasteurella multocida* PCR would also be an important method of further demonstrating the value of this assay. Dr Magne Bisgaard (Department of Veterinary Microbiology, The Royal Veterinary and Agricultural University, Copenhagen, Denmark) is the world's leading expert on *P. multocida* and related organisms. His laboratory holds an extensive collection of *P. multocida* and other members of the family *Pasteurellaceae*. A collaborative evaluation of the *P. multocida* PCR developed in this project using the extensive culture collection available in Dr Bisgaard's laboratory is currently being undertaken by Dr Blackall (funded by RIRDC under a travel grant award).

Intellectual Property

As the tests described in this report are based on the polymerase chain reaction, a technology that is already patented, and use gene sequences which are part of the normal bacterial cell, there are no issues related to intellectual property.

Communication Strategy

As outlined in the Recommendations section, the key users of the tests developed in this project are the diagnostic laboratory personnel that serve the Australian poultry industries. Hence, the most effective means of ensuring maximum commercial benefit to the Australian poultry industries is to ensure that results of this project are presented at industry meetings in Australia and as formal papers in the international scientific literature.

The following conference papers have already been presented:

Blackall, P.J., Miflin, J.K., Chen, X. and Zhang, P. 1995. Rapid detection of avian bacterial pathogens. Queensland Poultry Science Symposium, Gatton, Queensland.

Blackall, P.J., Bowles, R., Fegan, N., Miflin, J.K., Chew, G.T., Hampson, D.J., Chen, X. and Zhang, P. 1996. Towards the new frontier: an update on avian bacteriology research at the Animal Research Institute. Queensland Poultry Science Symposium, Gatton, Queensland.

Miflin, J.K. and Blackall, P.J. 1997. Development of a polymerase chain reaction for *Pasteurella multocida*. Scientific Meeting, Australian Veterinary Poultry Association, Melbourne, Victoria.

Miflin, J.K. and Blackall, P.J. 1997. Development of a polymerase chain reaction for *Pasteurella multocida*. Fourth Annual *Pasteurella* and Pasteurellosis Meeting, Dubbo, New South Wales.

Miflin, J.K., Blackall, P.J. and Chen, X. 1997. Rapid diagnostic tests for infectious coryza and fowl cholera. Queensland Poultry Science Symposium, Gatton, Queensland.

References

Abdullahi, M.Z., Gilmour, N.J.L. and Poxton, I.R. 1989. Identification of non-haemolytic pasteurellae cultured from the lungs of cattle. *Veterinary Record* 125:154.

Arzey, G. G. 1987. The effects of infectious coryza on the egg production of a layer flock. *Australian Veterinary Journal* 64:124-126.

Beveridge, W. I. B. and Hart, L. 1985. *Viral, bacterial and fungal diseases of poultry. Volume 7. Animal Health in Australia*. Australian Government Publishing Service, Canberra: p.154.

Bisgaard, M., Houghton, S.B., Mutters, R. and Stenzel, A. 1991. Reclassification of German, British and Dutch isolates of so-called *Pasteurella multocida* obtained from pneumonic calf lungs. *Veterinary Microbiology* 26:115-124.

Blackall, P.J., Chew, G.T.I., Fegan, N. and Hampson, D.J. 1996. Strain diversity and genetic structure of *Pasteurella multocida* infecting Australian poultry. Final report to Rural Industries Research and Development Corporation.

Blackall, P. J., Matsumoto, M. and Yamamoto, R. 1997. Infectious coryza. In *Diseases of Poultry* 10th edn, B.W. Calneck, H.J. Barnes, C.W. Beard, L.R. McDougald and Y.M. Saif (Eds), Iowa State University Press, Iowa: pp.179-190.

Carter, G.R. 1955. Serotyping of *Pasteurella multocida*. I. A hemagglutination test for the identification of serological type. *American Journal of Veterinary Research* 16:481-484.

Chen, X., Miflin, J. K., Zhang, P. and Blackall, P. J. 1996. Development and application of DNA probes and PCR tests for *Haemophilus paragallinarum*. *Avian Diseases* 40:398-407.

Dewhirst, F.E., Paster, B.J., Olsen, I. and Fraser, G.J. 1992. Phylogeny of 54 representative strains of species in the family *Pasteurellaceae* as determined by comparison of 16S rRNA sequences. *Journal of Bacteriology* 174:2002-2013.

Dewhirst, F.E., Paster, B.J., Olsen, I. and Fraser, G.J. 1993. Phylogeny of the *Pasteurellaceae* as determined by comparison of 16S ribosomal ribonucleic acid sequences. *Zentralblatt für Bakteriologie* 279:35-44.

Droual, R., Bickford, A. A., Charlton, B. R., Cooper, G. L. and Channing, S. E. 1990. Infectious coryza in meat chickens in the San Joaquin Valley of California. *Avian Diseases* 34:1009-1016.

Fegan, N., Blackall, P.J. and Pahoff, J.L. 1995. Phenotypic characterisation of *Pasteurella multocida* isolates from Australian poultry. *Veterinary Microbiology* 47:281-286.

Grimes, T. M. 1975. Causes of disease in two commercial laying hens. *Australian Veterinary Journal* 51:337-343.

Hart, L. 1938. The occurrence of fowl cholera in Australia. *Australian Veterinary Journal* 14:71-72.

Heddleston, K., Gallagher, J.B. and Rebers, P.A. 1972. Fowl cholera: gel diffusion precipitin test for serotyping *Pasteurella multocida* from avian species. *Avian Diseases* 16:925-936.

Hoerr, F. J., Putnam, M., Rowe-Rossmanith, S., Cowart, W. and Martin, J. 1994. Case report: Infectious coryza in broiler chickens in Alabama. *Proceedings 43rd Western Poultry Diseases Conference* California: p. 42.

Horner, R. F., Bishop, G. C., Jarvis, C. J. and Coetzer, T. H. T. 1995. NAD (V-factor)-independent and typical *Haemophilus paragallinarum* infection in commercial chickens: a five year field study. *Avian Pathology* 24:453-463.

Hungerford, T. G. (1968) A clinical note on avian cholera. *Australian Veterinary Journal* 44:31-32.

Jackson, C. A. W., Kingston, D. J. and Hemsley, L. A. 1972. A total mortality survey of nine batches of broiler chickens. *Australian Veterinary Journal* 48:481-487.

Kamp, E.M., Bokken, G.C.A.M., Vermeulen, T.M.M., de Jong, M.F., Buys, H.E.C.M., Reek, F.H., and Smits, M.A. 1996. A specific and sensitive PCR assay suitable for large-scale detection of toxicogenic *Pasteurella multocida* in nasal and tonsillar swabs specimens of pigs. *Journal of Veterinary Diagnostic Investigation* 8:304-309.

Kasten, R.W., Carpenter, T.E., Snipes, K.P. and Hirsh, D.C. 1997. Detection of *Pasteurella multocida*-specific DNA in turkey flocks by use of the polymerase chain reaction. *Avian Diseases* 41:676-682.

Lane, D. J. 1991. 16S/23S rRNA sequencing. In *Nucleic acid techniques in bacterial systematics*, E. Stackebrandt and M Goodfellow (Eds), John Wiley and Sons, Chichester: pp.115-175.

Madsen, E.B., Bisgaard, M., Mutters, R. and Pederson, K.B. 1985. Characterization of *Pasteurella* species isolated from lungs of calves with pneumonia. *Canadian Journal of Comparative Medicine* 49:63-67.

Mutters, R., Ihm, P., Pohl, S., Frederiksen, W. and Mannheim, W. 1985a. Reclassification of the genus *Pasteurella* Trevisan 1887 on the basis of deoxyribonucleic acid homology, with proposals for the new species *Pasteurella dagmatis, Pasteurella canis, Pasteurella stomatis, Pasteurella anatis,* and *Pasteurella langaa*. *International Journal of Systematic Bacteriology* 35:309-322.

Mutters, R., Piechulla, K., Hinz, K.-H. and Mannheim, W. 1985b. *Pasteurella avium* (Hinz and Kunjara 1977) comb. nov. and *Pasteurella volantium* sp. nov. *International Journal of Systematic Bacteriology* 35:5-9.

Reid, G. G. and Blackall, P. J. 1987. Comparison of adjuvants for an inactivated infectious coryza vaccine. *Avian Diseases* 31:59-63.

Reid, G. G., Grimes, T. M., Eaves, F. W. and Blackall, P. J. 1984. A survey of disease in five commercial flocks of meat breeder chickens. *Australian Veterinary Journal* 61:13-16.

Rhoades, K. R. and Rimler, R. B. 1991. Pasteurellosis. In *Diseases of Poultry* 10th edn, B.W. Calneck, H.J. Barnes, C.W. Beard, L.R. McDougald and Y.M. Saif (Eds), Iowa State University Press, Iowa: pp.145-162.

Rhoades, K. R., Rimler, R. B. and Sandhu, T. S. 1989. Pasteurellosis and Pseudotuberculosis. In *A Laboratory Manual for the Isolation and Identification of Avian Pathogens* , H.G. Purchase, L.H. Arp, C.H. Domermuth, and J.E. Pearson (Eds), American Association of Avian Pathologists, Pennsylvania: pp.14-21.

Sandoval, V. E., Terzolo, H. R. and Blackall, P. J. 1994. Complicated infectious coryza cases in Argentina. *Avian Diseases* 38:672-678.

Van Camp, G., Chapelle, S. and De Wachter, R. 1993. Amplification and sequencing of variable regions in bacterial 23S ribosomal RNA genes with conserved primer sequences. *Current Microbiology* 27:147-151.

Wilson, K.H., Blitchington, R.B. and Greene, R.C. 1990. Amplification of bacterial 16S ribosomal DNA with polymerase chain reaction. *Journal of Clinical Microbiology* 28:1942-1946.