PROJECT REPORT

Development of Molecular Tests for Serovar-Specific Identification and Typing of *Haemophilus paragallinarum*

DAQ 226 A

Final Report to Rural Industries Research and Development Corporation

P. J. BLACKALL[1] , M. I. KHAN[2], X. CHEN[3], C. SONG[3] and R. BOWLES[1]

[1]Department of Primary Industries, Queensland, Queensland Poultry Research and Development Centre, Animal Research Institute, Yeerongpilly 4105, Australia

[2]Department of Pathobiology, University of Connecticut, Storrs, Connecticut 06269, USA

[3]Institute for Animal Husbandry and Veterinary Science, Beijing Municipal Academy of Agriculture, Beijing 100081, People's Republic of China

Principal Investigator: Dr. P.J. Blackall, DPIQ, Queensland Poultry Research and Development Centre, Animal Research Institute, Locked Mail Bag 4, Moorooka, Q 4105, phone 07 3362 9498

Department of Primary Industries

Queensland

TABLE OF CONTENTS

EXECUTIVE SUMMARY

Two different types of rapid, DNA based typing methods were evaluated for their ability to recognise subtypes within the species *Haemophilus paragallinarum*. The two techniques were based on a molecular biology technique known as the polymerase chain reaction (PCR). One of the techniques was based on the use of random DNA sequences and is termed RAPD. The other technique is based on DNA sequences that were originally discovered in bacteria that belong to the family *Enterobacteriaceae*. This second technique is known as ERIC-PCR.

The two techniques were examined from two view points :-

A) ability to produce patterns that were serovar specific ie an ability to replace conventional serotyping;
B) ability to subtype below species and serovar level - allowing fingerprinting that is useful for disease outbreak investigations.

Despite extensive efforts, the RAPD technique could not be established to a stage where reproducible patterns could be obtained. The technique was thus abandoned.

ERIC-PCR did not give serovar specific patterns and thus cannot replace conventional serotyping.

However, the technique was shown to as effective as a typing tool for disease outbreak investigations. ERIC-PCR was shown to have the capacity to clearly distinguish unrelated isolates. The technique was also shown to be as good as some alternative molecular techniques for typing reference strains of *H. paragallinarum* and field isolates of *H. paragallinarum* from outbreaks of infectious coryza in China.

The very limited genetic diversity of Australian isolates of *H. paragallinarum*, first identified in previous RIRDC projects, was confirmed with ERIC-PCR. ERIC-PCR was unable to separate the 16 Australian isolates of *H. paragallinarum*. This study has shown that for Australian outbreaks of infectious coryza, the definitive typing technique remains the demanding and time-consuming technique of restriction endonuclease analysis.

This project has validated ERIC-PCR as a molecular typing tool for investigating outbreaks of infectious coryza. The technique, however, is of limited application in Australia because of the unique nature of the limited genetic diversity of Australian isolates of *H. paragallinarum*.

GENERAL PROJECT INFORMATION

Project Title: Development of Molecular Tests for Serovar-Specific Identification and Typing of *Haemophilus paragallinarum*

Project Number: DAQ 226 A

Background

The Disease - Infectious Coryza

Infectious coryza is an acute respiratory disease of chickens that may occur in both growing chickens and layers. The disease is world wide and causes economically significant problems in Central and South America, Africa, south east Asia, Japan, China, the Middle East, India, Germany, Australia and the United States (Blackall *et al.*, 1997).

The most common clinical signs are a nasal discharge, conjunctivitis, and swelling of the sinuses and face. Swelling of the wattles may develop also. Birds may have diarrhoea. Decreased feed and water consumption retards growth in young stock, and reduces egg production in laying flocks (Blackall *et al.*, 1997).

In modern intensive poultry industries based in countries with a generally high health standard, the major economic effect of the disease is an increased culling rate in meat chickens and a reduction in egg production (10 to 40%) in laying and breeding hens, particularly on multi-age farms (Blackall *et al.*, 1997). This drop in egg production can be significant even in flocks suffering an uncomplicated coryza outbreak that receives prompt antibiotic treatment. For example Arzey (1987) reported that such an outbreak in an Australian flock caused an egg drop that lasted 6 weeks with an average drop of 11.4% per week.

Recent Advances

Work performed recently at the Bacteriology Research Laboratory, funded in part by the RIRDC, has greatly improved our ability to diagnose infectious coryza. A species specific polymerase chain reaction (PCR) assay for *H. paragallinarum* has been developed (Chen *et al.*, 1996). This assay, based on a random cloned DNA fragment, is now being used for the routine diagnosis of infectious coryza at the Bacteriology Research Laboratory as well as at the collaborating institute in Beijing, China (The Institute for Animal Husbandry and Veterinary Science). The test has been shown to be as effective as culture when used on swabs taken from the sinuses of necropsied chickens (Chen *et al.*, 1996). In very recent work, the PCR has been shown to be superior to conventional culture, even when used under the typical field and laboratory conditions encountered in a developing country like China (Chen *et al.*, 1998).

In other work, the Bacteriology Research Laboratory, with funding from RIRDC and in collaboration with institutes in China and South Africa, has been developing finger-printing techniques for the fine typing of *H. paragallinarum* strains. Initially, the Bacteriology Research Laboratory developed the technique of restriction endonuclease analysis (REA) (Blackall *et al.*, 1991). REA has provided much useful information on the epidemiology of infectious coryza outbreaks, e.g. showing the key role of replacement stock in the spread of the disease (Blackall *et al.*, 1990b). In recent international collaborative work, the Bacteriology Research Laboratory has been using ribotyping to demonstrate that the unique NAD-independent strains of *H. paragallinarum* from South Africa are clonal in nature and appear to have developed from a point source (Miflin *et al.*, 1995). In other recent work, ribotyping has been used to demonstrate that a considerable diversity exists in the strains of *H. paragallinarum* circulating in China, despite the fact that all strains isolated to date have been a single serovar (Miflin *et al.*, 1997).

Some Key Remaining Problems

While the recent advances described above are major steps forward, several pressing problems remain. The new PCR test for *H. paragallinarum* described above does not provide any information on the serovar of *H. paragallinarum* isolates involved in an outbreak. In the widely used Page serotyping scheme, there are three serovars (A, B and C) of *H. paragallinarum* (Page, 1962). As inactivated vaccines protect only against those Page serovars in the vaccine (Blackall *et al.*, 1997), the implementation and monitoring of effective vaccination programmes is absolutely dependent upon a knowledge of the serovars present in the target chicken population. Serotyping of *H. paragallinarum* is a demanding procedure that requires considerable laboratory facilities as well as access to high titre hyper immune antisera (Blackall *et al.*, 1997). These limitations mean that the ability to serotype *H. paragallinarum* is available at only a few laboratories around the world, one of which is the Bacteriology Research Laboratory.

This limited availability of serotyping is a major restriction to the development of effective prevention and control programmes for infectious coryza. The replacement of the conventional serotyping by some form of DNA based test would be a major step forward, allowing many more laboratories to perform serotyping.

Another area where current technology is limiting advances is molecular epidemiology. While REA and ribotyping have been very useful (as described above), there are still major limitations with both approaches. REA analysis results in very complex banding patterns, making it difficult to compare strains or to quantitate differences among strains. While ribotyping overcomes these deficiencies, it is a time consuming technique that requires the isolation of high quality DNA and the performance of DNA hybridisation. A technique that avoids the requirement for the isolation of high purity DNA and that avoids the performance of hybridisation would be a major step forward.

Several studies have recently highlighted the potential for PCR based approaches for the development of DNA based tests at either the species level or the serovar level. As an example, Giesendorf *et al.* (1996) have recently reviewed the use of PCR-mediated fingerprinting for the development of both species-specific and strain specific DNA probes. They conclude that such an approach can quickly lead to a specific DNA-based tests, avoiding the need for extensive knowledge of the genes (and their sequences) that are responsible for such specificity (Giesendorf *et al.*, 1996). In another study, Hennessy *et al.* (1993) were able to replace conventional serotyping for *Actinobacillus pleuropneumoniae* with an arbitrarily primed (AP) PCR.

Similarly, there have been many studies highlighting the fact that PCR-based technologies such as AP-PCR offer a rapid method for the performance of genetic typing. Such typing has been used for a range of organisms e.g. *Mycoplasma synoviae*, *M. meleagridis* and *M. iowae* (Fan *et al.*, 1995) and *Campylobacter jejuni* (Lam *et al.*, 1995).

Objectives

A) Development of a DNA based alternative to the traditional serotyping of *H. paragallinarum*

B) Development of a rapid PCR-mediated fingerprinting technique for *H. paragallinarum*.

INTRODUCTION

Haemophilus paragallinarum is the causative agent of infectious coryza, an upper respiratory tract disease of chickens characterised by relatively mild clinical signs (Blackall *et al.*, 1997). The economic impact of the disease is attributed to the resultant increase in unthrifty chickens and significant reductions (10%-40%) in egg production (Blackall *et al.*, 1997).

There have been few studies on methods to type isolates of *H. paragallinarum*. Most typing of *H. paragallinarum* has involved the use of two of the three recognised serotyping schemes - the Page scheme (Page, 1962) and the modified Kume scheme (Kume *et al.*, 1983; Blackall *et al.*, 1990a). This use of serological typing requires access to specific, high titre antisera - a major limitation. Alternative methods such as carbohydrate fermentation patterns and antibiotic resistance patterns have been shown to yield few subtypes (Blackall *et al.*, 1989). Both whole-cell proteins and outer-membrane proteins have been shown to exist in only two major banding patterns (Blackall and Yamamoto, 1989; Blackall *et al.*, 1990c).

The limitations outlined above for the traditional phenotypic typing methods have been well recognised in many bacterial species. Hence, molecular fingerprinting methods have been developed for many bacterial species, including *H. paragallinarum*. Restriction endonuclease analysis (REA) has been shown to yield patterns that are stable *in vitro* and *in vivo* (Blackall *et al.*, 1990b, 1991). REA has proven useful in examining the epidemiology of outbreaks of infectious coryza (Blackall *et al.*, 1990b). Ribotyping has also proven useful - confirming that the recently recognised V-factor independent isolates of *H. paragallinarum* found in South African poultry are clonal (Miflin *et al.*, 1995) and demonstrating the diversity amongst a collection of Chinese *H. paragallinarum* isolates (Miflin *et al.*, 1997). The limitation of the molecular fingerprinting methods that have been used for *H. paragallinarum* is that both methods (REA and ribotyping) are time-consuming, technically demanding and require high quality DNA. Polymerase chain reaction (PCR) typing methods are far more rapid (Appuhamy *et al.*, 1997). As well, PCR-based fingerprinting techniques generate banding patterns that contain fewer bands than that typically present in REA patterns, which often contain over 100 bands. Hence, PCR based fingerprinting techniques have distinct attractions over the alternative molecular fingerprinting methods. In this paper, we report on a evaluation of a PCR based fingerprinting methods for the typing of *H. paragallinarum*.

Two variations of PCR fingerprinting were used in this study - RAPD (random amplification polymorphic DNA) and ERIC-PCR (a PCR based on primers that target enterobacterial repetitive insertion consensus sequences). We report on the ability of both types of PCR to A) generate serovar specific patterns and B) generate epidemiologically relevant typing below the species level.

MATERIALS AND METHODS

Bacteria

The eleven reference strains for the Page and Kume serotyping schemes (Table 1) were used. A total of 25 field isolates of *H. paragallinarum*, 16 from Australian chickens (Table 2) and nine from Chinese chickens (Table 3) were also used. The Australian isolates were previously shown to be *H. paragallinarum*, either Page serovar A/Kume serovar A-4 (three isolates) or Page serovar C/Kume serovar C-2 (13 isolates) (Eaves *et al.*, 1989). The reference strains and the Australian field isolates have all been typed by multilocus enzyme electrophoresis (MLEE) and double enzyme ribotyping using the enzymes *Hpa*II and *Ssp*I (Blackall and Bowles, 1996). The Australian field isolates have been the subject of a detailed epidemiological study, involving both field investigation as well as molecular fingerprinting by restriction endonuclease analysis (REA) (Blackall *et al.*, 1990b). The nine Chinese isolates have been shown to be *H. paragallinarum* Page serovar A (Miflin *et al.*, 1997; Chen *et al.*, 1998).

Media

Test medium agar supplemented with chicken serum and NADH, prepared as described (Reid and Blackall, 1987), was used for the routine cultivation of all isolates. Agar plates were incubated at 37^{o}C under 5% CO_2. A liquid version of this medium, test medium broth (TMB), was also used. TMB was incubated aerobically.

DNA Extraction

The cultures were grown overnight at 37^{o}C in TMB and harvested by centrifugation. For the serovar reference strains and the Australian field isolates, chromosomal DNA was extracted as described previously (Fadl *et al.*, 1995). For the Chinese isolates, chromosomal DNA was extracted as described (Blackall *et al.*, 1995). DNA concentration and purity were determined spectrophotometrically.

RAPD

For RAPD amplification, the following conditions, originally recommended by Williams *et al.* (1990), were used. Amplification reactions were performed in a volume of 25 µl containing 10 mM Tris-Cl, pH 8.3, 50 mM KCl, 2 mM MgCl2, 0.001% gelatin, 100 µM each of dATP, dCTP, dGTP, and dTTP, 5 picomoles of a single 10-base primer, 25 ng of genomic DNA, and 0.5 units of Taq DNA polymerase. Amplification was done in a thermocycler (Hybaid, Touchdown). The amplification process consisted of 45 cycles of 1 min at 94°C, 1 min at 36°C, and 2 min at 72°. Amplified products were separated by horizontal electrophoresis through a 1.5% agarose gel at 40V for 2-3 h in Tris-Borate-EDTA electrophoresis buffer (Sambrook *et al.*, 1989). A 1 kb DNA ladder (Gibco-BRL) was used as a marker.

The 10 mer primers used in this study were primers 1, 6, 7, 10, 11, 13 and 20 from the Operon 10 Mer Primer Kit A (Operon Technologies). The actual sequences of the primers was as follows:-

Primer 1	CAGGCCCTTC
Primer 6	GGTCCCTGAC
Primer 7	GAAACGGGTG
Primer 10	GTGATCGCAG
Primer 11	CAATCGCCGT
Primer 13	CAGCACCCAC
Primer 20	GTTGCGATCC

In comparing RAPD profiles, isolates were assigned to profiles on the basis of having at least one different band.

ERIC-PCR

The primers used for ERIC-PCR were ERIC-1R (ATGTAAGCTCCTGGGGATTCAC) and ERIC-2 (AAGTAAGTGACTGGGGTGAGCG) (Versalovic *et al.*, 1991). The 50 µl reaction mixture contained 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 3 mM $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphate, 100 pM of each primer, 0.5 U of *Taq* DNA polymerase and 2.5 µl of template DNA (200 ng of DNA). Amplification was done in a thermocycler (Hybaid, Touchdown). The amplification process consisted of an initial denaturation at 95^0C for 5 min, then 35 cycles consisting of denaturation at 94^0C for 30 s, annealing at 50^0C for 30 s, and extension at 72^0C for 6 min, with a final extension at 72^0C for 6 min. Amplified products were separated by horizontal electrophoresis through a 1.5% agarose gel at 40V for 2-3 h in Tris-Borate-EDTA electrophoresis buffer (Sambrook *et al.*, 1989). A 1 kb DNA ladder (Gibco-BRL) was used as a marker.

In comparing ERIC-PCR profiles, isolates were assigned to profiles on the basis of having at least one different band. Each different ERIC-PCR profile was assigned a unique number.

Ribotyping

The Chinese isolates were ribotyped using a method previously described (Miflin *et al.*, 1997). Briefly, total DNA (5 ug) was digested separately with each of the restriction endonucleases *Hae*III and *Hpa*II (Boehringer Mannheim, Mannheim, Germany) in Tris-acetate (TA) buffer (O'Farrell *et al.*, 1980) using 10 U enzyme per 50 ml reaction. The reaction was performed at 37^oC for 3 h and terminated by heating at 65^oC for 20 min. DNA fragments were separated by horizontal electrophoresis through a 0.7% agarose gel at 25 V for 16 h in Tris-Borate-EDTA electrophoresis buffer (Sambrook *et al.*, 1989). Digoxigenin-labelled DNA Molecular-weight Marker II (Boehringer Mannheim) was used as a marker. DNA from the agarose gel was transferred to a positively charged nylon membrane (Boehringer Mannheim) with the VacuGene XL Vacuum Blotting System (Pharmacia LKB Biotechnology, Uppsala, Sweden) according to the manufacturer's instructions. DNA was bound to the membrane by ultraviolet cross-linking at 254 nm for 3 min.

The probe consisted of a PCR-generated 16S rDNA fragment of the type strain of *H. paragallinarum* (ATCC 29545), amplified with the universal primers 27f and 1525r (Lane, 1991). This represents an almost complete copy of the double stranded 16S rRNA gene

sequence of *H. paragallinarum* strain ATCC 29545. Before use the probe was labelled with digoxigenin using the DIG DNA Labeling Kit (Boehringer Mannheim). The concentration of the labelled probe was determined by comparison with labelled control DNA provided in the kit, using the manufacturer's protocol. The probe was used at a concentration of 15-20 ng per ml of hybridisation solution.

Southern hybridisation and detection were performed as described in the Nucleic Acid Detection Kit (Boehringer Mannheim) using a temperature of 68^{o}C for both pre-hybridisation and hybridisation steps and post-hybridisation washes.

In comparing ribotyping profiles, isolates were assigned to profiles on the basis of having at least one different band.

RESULTS

RAPD

The evaluation of RAPD was performed using the 11 reference strains of the Page and Kume serotyping schemes.

Despite repeated efforts, none of the seven different RAPDs performed using the seven available primers gave consistent banding patterns. Further, no RAPD based on various possible combinations of double primers gave any consistent banding pattern.

ERIC-PCR

Amongst the 11 reference strains and 25 field isolates used in this study, a total of 17 different ERIC-PCR patterns were recognised (Tables 1, 2 and 3). An example of some of the different pattern types is shown in Fig. 1.

All 11 reference strains for the Page and Kume serovars showed unique ERIC-PCR patterns except the two Australian isolates (HP14 and HP60) which showed the same pattern. This pattern for Australian reference strains HP14 and HP60 was also possessed by all 16 of the Australian field isolates. Both MLEE and ribotyping had been previously used to type 10 of the 11 reference strains examined by ERIC-PCR. By ERIC-PCR, all ten of these strains gave unique patterns. MLEE typing assigned eight of the 10 strains to unique but grouped strains Modesto and HP14 together. With the same 10 reference strains, ribotyping allocated seven strains to unique types but grouped strains 0222, IPDH 2403 and IPDH 2671 together. The available knowledge of serovars and source of origin indicates that all 10 reference strains examined by MLEE, ribotyping and ERIC-PCR should give unique, distinct patterns.

The 16 Australian field isolates all had the same ERIC-PCR pattern despite belonging to two different Page/Kume serovars and three different restriction endonuclease (REA) profiles (Table 2). The available evidence indicates that subtyping achieved by REA matches the epidemiology of the outbreaks centred on these isolates (Blackall *et al.*, 1990b). The results of the ERIC-PCR corresponded with the MLEE typing - both techniques allocated all 16 isolates to the same pattern. Both techniques also allocated the Australian reference strain HP14 to the same type as these field isolates. Ribotyping recognised considerably more types within the Australian field isolates (6 types) than MLEE, ERIC-PCR (both gave 1 type only) or REA (three types).

A total of seven different ERIC-PCR patterns were recognised amongst the nine Chinese isolates. In contrast, ribotyping, performed using a combination of both *Hae*III and *Hpa*II recognised eight patterns. There was a strong, but not perfect, correlation between the results of the ribotyping and ERIC-PCR. Both ribotyping and ERIC-PCR suggested isolates HP558 and HP560, obtained from different farms in Hebei province were the same. Ribotyping suggested that isolates HP559, HP564 and HP574 were all unique - a finding supported by ERIC-PCR. Isolates HP561 and HP353 shared a common ribotyping pattern but showed different ERIC-PCR fingerprints. Isolates HP562 and HP569 were different by ribotyping but shared a common ERIC-PCR fingerprint. Both methods, ribotyping and

ERIC-PCR, showed that the only two isolates obtained from the same farm (HP564 and HP569) were different.

Table 1. *H. paragallinarum* reference strains used in the study

Strain	Serovar Page/Kume	Country	Source[A]	MLEE Type[B]	Ribotype[B]	ERIC-PCR Type[C]
0083	A/A-1	USA	Rimler	2	17	2
0222	B/B-1	USA	Rimler	10	31	3
Modesto	C/C-2	USA	Rimler	1	2	4
JAP 221	A/A-1	Japan	Kume	4	18	6
IPDH 2403	A/A-2	Germany	Hinz	13	31	5
E3C	A/A-3	Japan	Kume	8	30	7
HP14	A/A-4	Australia	Own isolate	1	1	1
IPDH 2671	B/B-1	Germany	Kume	14	31	10
H-18	C/C-1	Japan	Kume	15	36	9
SA-3	C/C-3	Africa	Kume	6	28	8
HP60	C/C-4	Australia	Own isolate	ND	ND	1

[A] Kume = Dr. K. Kume, The Kitasato Institute, Japan; Rimler = Dr. R. Rimler, National Animal Disease Centre, Ames, Iowa, USA

[B] As previously defined by Blackall and Bowles (1996)

[C] As defined in this study

Table 2. Australian isolates of *H. paragallinarum* used in the study

Isolate	Serovar (Page/Kume)	Outbreak (Farm)	REA Type	MLEE Type[A]	Ribotype[A]	ERIC-PCR Type[B]
HP167	C/C-2	1 (A)	I	1	6	1
HP173	C/C-2	1 (A)	I	1	7	1
HP177	C/C-2	1 (B)	I	1	7	1
HP181	C/C-2	2 (A)	I	1	6	1
HP183	C/C-2	2 (A)	I	1	10	1
HP192	C/C-2	2 (A)	I	1	10	1
HP193	C/C-2	2 (A)	I	1	10	1
HP187	C/C-2	2 (C)	I	1	6	1
HP188	C/C-2	2 (D)	I	1	6	1
HP185	C/C-2	2 (E)	I	1	6	1
HP180	C/C-2	2 (F)	I	1	9	1
HP166	C/C-2	3 (G)	II	1	2	1
HP186	C/C-2	5 (I)	II	1	2	1
HP179	A/A-4	4 (H)	III	1	8	1
HP189	A/A-4	4 (H)	III	1	2	1
HP184	A/A-4	6 (J)	III	1	2	1

[A] As previously defined by Blackall and Bowles (1996)

[B] As defined in this study

Table 3. Chinese isolates of *H. paragallinarum* used in the study

Isolate	Page Serovar	Province/Farm	Ribotype[A]	ERIC-PCR Type[A]
HP558	A	Hebei/A	I	11
HP559	A	Hebei/B	II	12
HP560	A	Hebei/C	I	11
HP353	A	Hebei/D	III	17
HP561	A	Yunnan/A	III	13
HP562	A	Yunnan/B	IV	14
HP564	A	Shandong/A	V	15
HP569	A	Shandong/A	VI	14
HP574	A	Shandong/B	VII	16

[A] As defined in this study

Figure 1. ERIC-PCR profiles for selected reference strains and field isolates of *H. paragallinarum*. Lane 1 = Molecular Weight Marker; lane 2 = 0083, type 2; lane 3 = Modesto, type 4; lane 4 = HP14, type 1, lane 5 = IPDH 2403, type 5; lane 6 = E3C, type 7; lane 7 = HP558, type 11; lane 8 = HP559, type 12; lane 9 = HP561, type 13; lane 10 = HP562, type 14; lane 11 = HP166, type 1; lane 12 = HP167, type 1; lane 13 = HP181, type 1; lane 14 = HP187, type 1, lane 15 = HP193, type 1.

DISCUSSION

One of the main objectives of this project was to develop a DNA based alternative to the traditional serotyping of *H. paragallinarum*. This objective was not achieved. The RAPD technique, including all seven different primer variations, failed to give consistent banding patterns. It is possible that more extensive investigations may eventually find primers that are suitable for use in RAPDs with *H. paragallinarum*. Such investigations were beyond the time scale available for this study (six months). Hence, it was decided to abandon the RAPD approach.

This failure to establish a working RAPD technique greatly limited the possibilities of developing a serovar-specific DNA test. While consistent and reproducible banding patterns were produced by ERIC-PCR, the resultant typing patterns did not convey any information on serotyping. This was particularly evident with the ERIC-PCR patterns obtained with the Australian field isolates and reference strains. These 18 strains/isolates represented three Kume serovars C-2, C-4 and A-4 - yet all gave a single ERIC-PCR pattern.

The second objective of this study was the development of a PCR-based fingerprinting technique suitable for use as a typing tool for *H. paragallinarum*. The failure of the RAPD approach meant that the development and validation of a typing technique concentrated on ERIC-PCR.

There are two important features of a typing technique - A) sufficient sensitivity to distinguish unrelated isolates and B) sufficient specificity to group all related isolates. These criteria form the basis of the evaluation of ERIC-PCR typing described in this study. The evaluation was performed by comparing ERIC-PCR with three other methods of typing - REA, ribotyping and MLEE typing. Both REA and ribotyping have been validated for use with *H. paragallinarum* in previous studies at the Animal Research Institute - showing an ability to distinguish unrelated isolates and to group related isolates (Blackall *et al.*, 1990b, 1991; Miflin *et al.*, 1995, 1997). While MLEE typing has not been previously used with *H. paragallinarum*, it has been shown to provide valuable insights into the overall population structure of *H. paragallinarum* (Blackall and Bowles, 1996). As well, MLEE typing has been shown to be a useful typing technique in other bacteria e.g. *Campylobacter* (Patton *et al.*, 1991) and *Listeria* (Boerlin and Piffaretti, 1991).

To evaluate ERIC-PCR in terms of an ability to separate diverse strains, all the reference strains for the two major serotyping schemes for *H. paragallinarum* (the Kume and Page serotyping schemes) were examined - a total of 11 strains. All 11 strains have no known epidemiological connection and only four of the 11 strains belonged to the same Kume serovar - 0083 and IPDH belonging to Kume serovar A-1 and 0222; IPDH2671 belonging to Kume serovar B-1. As the strains involved in these shared serovars are from different continents, it is reasonable to assume that these strains, like all the others, should be distinct and unrelated. Hence an effective typing technique, when applied to these reference strains, should find all 11 strains to be different.

To evaluate the ability of the techniques to group related isolates, we used two sets of field isolates. One set of isolates was obtained from a previous detailed epidemiological

investigation of six outbreaks of infectious coryza in northern New South Wales (Blackall *et al.,* 1990b). The second set of field isolates were recently collected from field outbreaks of coryza in China. We used two sets of field isolates as there is considerable evidence that Australian isolates of *H. paragallinarum* show a unique and very limited genetic diversity (Blackall and Bowles, 1996).

ERIC-PCR performed well in terms of ability to distinguish clearly unrelated strains - 10 unique ERIC-PCR patterns were found amongst the 11 serovar reference strains. The only strains sharing a common pattern were the two Australian isolates - HP14 and HP60. The comparison of ERIC-PCR with MLEE and ribotyping was done using 10 of the 11 reference strains - all 10 having a unique ERIC-PCR fingerprint. Both MLEE and ribotyping were less discriminatory than ERIC-PCR (9 unique patterns amongst the 10 strains for MLEE and 8 unique patterns amongst the 10 strains for ribotyping).

The failure of ERIC-PCR to distinguish the two Australian reference strains is not a surprising finding. There is already considerable evidence that Australian isolates of *H. paragallinarum* are uniquely limited in genetic diversity (Blackall and Bowles, 1996).

In terms of ability to group related isolates, ERIC-PCR did not perform well with the Australian field isolates (see Table 2), failing to separate the 16 field isolates into the three recognised outbreak groupings. Similarly, MLEE typing, which like ERIC-PCR assigned all 16 isolates to one type, also performed poorly. While ribotyping was able to recognise a number of types in the Australian field isolates (five in total), the groupings recognised did not match the known epidemiological groupings. As an example, ribotyping suggested that eight isolates from Outbreak 2 consisted of three different ribotypes - 6, 9 and 10. This grouping is not supported by the field information or REA typing (Blackall *et al.*, 1990b).

When used on the Chinese field isolates, ERIC-PCR recognised a number of types (seven types in the nine isolates). The typing achieved by ERIC-PCR matched the available field knowledge and showed a good correlation with ribotyping. Both ERIC-PCR and ribotyping grouped the two isolates from Farms A and C in Hebei. Similarly, both ERIC-PCR and ribotyping allocated the two isolates from Farm G in Shandong to two different types.

There are significant differences in the practicalities of the various typing techniques used in this study. REA is by far the most demanding of the typing techniques. The demanding nature of REA is caused by the complexity of the patterns detected, frequently over 100 bands. Comparing isolates by REA requires high quality DNA to ensure complete digestion and excellent electrophoretic technique. These are demanding conditions, as reflected by the fact to date that only one laboratory in the world (the Bacteriology Research Laboratory) has published reports on the use of REA with *H. paragallinarum*. Overall, while yielding excellent results, REA is not suitable for practical use.

MLEE is a technique that has not moved out of research laboratories. It requires specialised reagents and equipment that are not widely available. Like REA, the Bacteriology Research Laboratory is the only laboratory to publish papers on the use of MLEE with *H. paragallinarum*. Again, for practical reasons, MLEE is not suited for routine use in typing studies.

Ribotyping, like REA, requires high quality DNA and good electrophoretic technique. It has an additional step (DNA hybridisation) after gel electrophoresis, adding an extra day to the time needed to obtain results. Ribotyping also requires, for practical application, two additional pieces of equipment (blotting apparatus and hybridisation oven) that are not normally available in routine laboratories.

ERIC-PCR can be performed within the same time-frame as REA and, hence, in a shorter time frame than ribotyping. There is a need for one specialised piece of equipment, a thermocycler. However, thermocyler technology is now becoming central to many diagnostic assays, both in-house developed assays and commercial kits. For this reason, thermocyclers are now commonly available in many diagnostic laboratories. ERIC-PCR generates banding patterns that are more complex than those typically seen with ribotyping. However, the patterns are considerably easier to examine than the typical REA patterns. Overall, ERIC-PCR typing is a rapid and practical method that is suited to most modern well equipped laboratories. As the current study was an initial investigation and development, we used purified DNA. However, other studies have demonstrated that ERIC-PCR assays can be performed directly on bacterial colonies without the need for DNA extraction (Chatelut *et al.*, 1995; Liu *et al.*, 1995). Hence, it should be possible to perform ERIC-PCR typing within 24 hr of obtaining a pure culture - much shorter than REA or ribotyping (typically 3 days and 4 days respectively).

This study has shown that ERIC-PCR can be a useful technique for typing *H. paragallinarum*. ERIC-PCR outperformed both ribotyping and MLEE typing in separating the Page and Kume serovar reference strains. ERIC-PCR performed as well as ribotyping when used on the Chinese field isolates. ERIC-PCR failed to separate any of the 16 Australian field isolates or the two Australian serovar reference strains. Indeed, only REA appears to be a suitable technique for typing Australian isolates of *H. paragallinarum* as MLEE failed to distinguish amongst these isolates while the grouping achieved by ribotyping did not reflect the known epidemiological groupings amongst the field isolates.

TECHNICAL SUMMARY

Two forms of polymerase chain reaction (PCR) fingerprinting were evaluated for their ability to subtype isolates of *Haemophilus paragallinarum* - random amplification polymorphic DNA (RAPD) and ERIC-PCR (a PCR based on primers that target enterobacterial repetitive insertion consensus sequences).

The two techniques were examined from two view points :-
A) ability to produce patterns that were serovar specific ie an ability to replace conventional serotyping;
B) ability to subtype below species and serovar level - allowing fingerprinting that is useful for disease outbreak investigations.

The tests were evaluated using three sets of *H. paragallinarum*. One set consisted of the 11 reference strains for the two main serotyping schemes of *H. paragallinarum* - the Page and Kume schemes. The second set consisted of 16 Australian field isolates that had been the subject of a previous detailed epidemiological study. The third set consisted of nine Chinese field isolates of *H. paragallinarum*. Using these sets of organisms, ERIC-PCR was compared with three other alternative typing techniques - restriction endonuclease (REA), ribotyping and multi-locus enzyme electrophoresis (MLEE).

RAPD was performed using seven different, random, 10 mer primers. Despite extensive efforts, the RAPD technique could not be established to a stage where reproducible patterns could be obtained. The technique was thus abandoned.

ERIC-PCR did not give serovar specific patterns and thus cannot replace conventional serotyping.

However, ERIC-PCR was shown to as effective as a typing tool - yielding a total of 17 different patterns among the 11 reference strains and 25 field isolates of *H. paragallinarum* used in the study.

The 11 reference strains were used to assess the ability of ERIC-PCR to separate clearly unrelated organisms. ERIC-PCR performed well in this regard - 10/11 reference strains had different ERIC-PCR patterns. The only two reference strains to share a common pattern were both Australian. ERIC-PCR performed slightly better at differentiating these reference strains than the alternative techniques of ribotyping and MLEE (nine and eight patterns amongst 10 strains, respectively).

ERIC-PCR failed to differentiate amongst the 2 Australian reference strains or the 16 field isolates - allocating all 18 to a single pattern type. These 18 isolates/strains represented three Kume serovars, two Page serovars and at least three different REA patterns. Hence, ERIC-PCR is not an efficient technique for typing Australian isolates of *H. paragallinarum*. Of the alternative typing techniques, MLEE also failed to separate the Australian isolates/strains while ribotyping achieved too fine a level of typing - yielding groupings that did not match the known epidemiology of the field isolates. The failure of ERIC-PCR to separate the Australian isolates/strains is probably due to the very limited

genetic diversity of Australian isolates of *H. paragallinarum*, a phenomenon first identified in previous RIRDC projects. This study has shown that for Australian outbreaks of infectious coryza, the definitive typing technique remains the demanding and expensive technique of restriction endonuclease analysis.

When used to investigate nine isolates of *H. paragallinarum* obtained from Chinese chickens, ERIC-PCR recognised seven different pattern types. The typing achieved by ERIC-PCR was supported by ribotyping (although the methods were not in total agreement) and the known epidemiology of the Chinese isolates.

Overall, this study has validated ERIC-PCR as a molecular typing tool for investigating outbreaks of infectious coryza. The technique, however, is of limited application in Australia because of the unique nature of the limited genetic diversity of Australian isolates of *H. paragallinarum*.

REFERENCES

Appuhamy, S., Parton, R., Coote, J. G. and Gibbs, H. A. (1997) Genomic fingerprinting of *Haemophilus somnus* by a combination of PCR methods. *Journal of Clinical Microbiology* **35**:288-291

Arzey, G. G. (1987) The effects of infectious coryza on the egg production of a layer flock. *Aust. Vet. J.* **64**:124-126

Blackall, P. J. and Bowles, R. (1996): Final Report to RIRDC - Population Studies on Bacterial Poultry Pathogens.

Blackall, P. J. and Yamamoto, R. (1989) Whole-cell protein profiles of *Haemophilus paragallinarum* as detected by polyacrylamide gel electrophoresis. *Avian Dis.* **33**:168-173

Blackall, P. J., Eaves, L. E. and Rogers, D. G. (1989) Biotyping of *Haemophilus paragallinarum* by hemagglutination serotyping, and carbohydrate fermentation, and antimicrobial drug resistance patterns. *Avian Dis.* **33**:491-496

Blackall, P. J., Eaves, L. E. and Rogers, D. G. (1990a) Proposal of a new serovar and altered nomenclature for *Haemophilus paragallinarum* in the Kume hemagglutinin scheme. *J. Clin. Microbiol.* **28**:1185-1187

Blackall, P. J., Morrow, C. J., McInnes, A., Eaves, L. E. and Rogers, D. G. (1990b) Epidemiologic studies on infectious coryza outbreaks in northern New South Wales, Australia, using serotyping, biotyping, and chromosomal DNA restriction endonuclease analysis. *Avian Dis.* **34**:267-276

Blackall, P. J., Rogers, D. G. and Yamamoto, R. (1990c) Outer-membrane proteins of *Haemophilus paragallinarum*. *Avian Dis.* **34**:871-877

Blackall, P. J., Eaves, L. E. and Morrow, C. J. (1991) Comparison of *Haemophilus paragallinarum* isolates by restriction endonuclease analysis of chromosomal DNA. *Vet. Microbiol.* **27**:39-47

Blackall, P. J., Pahoff, J. L., Marks, D., Fegan, N. and Morrow, C. J. (1995) Characterisation of *Pasteurella multocida* isolates from fowl cholera outbreaks on seven turkey farms. *Aust. Vet. J.* **72**:135-138

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

Boerlin, P. and Piffaretti, J.-C. (1991) Typing of human, animal, food, and environmental isolates of *Listeria monocytogenes* by multilocus enzyme electrophoresis. *Appl. Environ. Microbiol.* **57**:1624-1629

Chatelut, M., Dournes, J. L., Chabanon, G. and Marty, N. (1995) Epidemiological typing of *Stenotrophomonas (Xanthomonas) amltophilia* by PCR. *Journal of Clinical Microbiology* **33**:912-914

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 Dis.* **40**:398-407

Chen, X., Chen, Q., Zhang, P., Feng, W. and Blackall, P. J. (1998) Evaluation of a PCR test for the detection of *Haemophilus paragallinarum* in China. *Avian Pathol.* **28** (in press).

Eaves, L. E., Rogers, D. G. and Blackall, P. J. (1989) Comparison of hemagglutinin and agglutinin schemes for the serological classification of *Haemophilus paragallinarum* and proposal of a new hemagglutinin serovar. *J. Clin. Microbiol.* **27**:1510-1513

Fadl, A. A., Nguyen, A. V. and Khan, M. I. (1995) Analysis of *Salmonella enteritidis* by arbitrarily primed PCR. *Journal of Clinical Microbiology* **33**:987-989

Fan, H. H., Kleve, S. H. and Jackwood, M. W. (1995) Studies of intraspecies heterogeneity of *Mycoplasma synoviae, M. meleagridis*, and *M. iowae* with arbitrarily primed polymerase chain reaction. *Avian Dis.* **39**:766-777

Giesendorf, B. A. J., Quint, W. G. V., Vandamme, P. and Van Belkum, A. (1996) Generation of DNA probes for detection of microorganisms by polymerase chain reaction fingerprinting. *Zbl. Bakt.* **283**:417-430

Hennessy, K. J., Iandolo.JJ and Fenwick, B. W. (1993) Serotype identification of *Actinobacillus pleuropneumoniae* by arbitarily primed polymerase chain reaction. *J. Clin. Microbiol.* **31**:1155-1159

Kume, K., Sawata, A., Nakai, T. and Matsumoto, M. (1983) Serological classification of *Haemophilus paragallinarum* with a hemagglutinin system. *J. Clin. Microbiol.* **17**:958-964

Lam, K. M., Yamamoto, R. and DaMassa, A. J. (1995) DNA diversity among ioslates of *Campylobacter jejuni* detected by PCR-based RAPD fingerprinting. *Vet. Microbiol.* **45**:269-274

Lane, D. J. (1991) 16S/23S rRNA sequencing. *Nucleic acid techniques in bacterial systematics* (Eds.), John Wiley & Sons, Chichester, pp. 115-175.

Liu, P. Y.-F., Lau, Y.-J., Hu, B.-S., Shyr, J.-M., Shi, Z.-Y., Tsai, W.-S., Lin, Y.-H. and Tseng, C.-Y. (1995) Analysis of clonal relationships among isolates of *Shigella sonnei* by different molecular typing methods. *Journal of Clinical Microbiology* **33**:1779-1783

Miflin, J. K., Horner, R. F., Blackall, P. J., Chen, X., Bishop, G. C., Morrow, C. J., Yamaguchi, T. and Iritani, Y. (1995) Phenotypic and molecular characterization of V-factor (NAD)-independent *Haemophilus paragallinarum*. *Avian Dis.* **39**:304-308

Miflin, J. K., Chen, X. and Blackall, P. J. (1997) Molecular characterisation of isolates of *Haemophilus paragalinarum* from China by ribotyping. *Avian Pathol.* **27**:119-127

O'Farrell, P. H., Kutter, E. and Nakanishi, M. (1980) A restriction map of the bacteriophage T4 genome. *Mol. Gen. Genet.* **179**:421-435

Page, L. A. (1962) *Haemophilus* infections in chickens. 1. Characteristics of 12 *Haemophilus* isolates recovered from diseased chickens. *Am. J. Vet. Res.* **23**:85-95

Patton, C. M., Wachsmuth, L. K., Evins, G. M., Kiehlbauch, J. A., Plikaytis, B. D., Troup, N., Tompkins, L. and Lior, H. (1991) Evaluation of 10 methods to distinguish epidemic-associated *Campylobacter* strains. *J. Clin. Microbiol.* **29**:680-688

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

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*. . 2nd. Cold Spring Harbour Laboratory Press, Cold Spring Harbour, NY, 2000.

Versalovic, J., Koeuth, T. and Lupski, J. R. (1991) Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. *Nucleic Acids Res.* **19**:6823-6831

Williams, J. G. K., Kubelik, A. R., Livak, K. J., Rafalski, J. A. and Tingey, S. V. (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.* **18**:6531-6535