# Characterisation of *Pasteurella multocida* isolated from fowl cholera outbreaks on turkey farms

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**SUMMARY: Biochemical profiles, restriction endonuclease analysis (REA) and ribotyping were used to investigate *Pasteurella multocida* isolates from outbreaks of fowl cholera on 7 turkey farms in New South Wales. While only a single isolate was available from 5 of the farms, multiple isolates, 4 and 12 respectively, were available from the other 2 farms. The available field evidence suggested that 8 outbreaks had occurred with one farm suffering 2 outbreaks. The isolates obtained were all confirmed as *Pasteurella multocida*. Biochemical profiles allocated the isolates to 4 groups, 3 being variants of *P multocida* subsp *multocida* and the fourth being *P multocida* subsp *septica*. REA performed with *Hpa*II established 7 groups. Ribotyping using the *Hpa*II digests probed with the 16S rRNA operon of *Haemophilus paragallinarum* recognised the same 7 groups as REA. Unlike the biochemical profiles, both REA and ribotyping provided a fine subdivision that identified outbreaks as either related or unrelated. The REA and ribotyping patterns as well as biochemical profiles were stable for all isolates from the outbreaks in which multiple isolates were obtained from either the same bird or from different birds. REA and ribotyping were found to be superior to biotyping methods for the investigation of fowl cholera outbreaks.**

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## Introduction

Fowl cholera is a common and widely distributed disease of poultry and is of major economic importance (Rhoades *et al* 1989). While all species of birds are affected, turkeys are particularly susceptible (Rhoades *et al* 1989). The disease is caused by the bacterium *Pasteurella multocida* (Rhoades *et al* 1989).

*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 and its epidemiology. In particular, Mutters *et al* (1985) 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*. Molecular techniques such as restriction endonuclease analysis (REA) and ribotyping have enabled workers to distinguish strains of *P multocida* as a method of studying outbreaks of fowl cholera (Christiansen *et al* 1992a).

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 meat breeder hens and chickens, 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. In the only serological characterisation study performed in Australia, Ireland *et al* (1989) reported that over 75% of 65 isolates of *P multocida* from Australian chickens were serovars 1, 3 or 3 × 4.

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There appears to have been no previous report on the use of molecular techniques in the investigation of fowl cholera outbreaks in Australian poultry. In this paper, we report a study of isolates derived from 8 fowl cholera outbreaks on 7 turkey farms. We describe our application of the new extended phenotypic tests for the subspeciation of *P multocida* and also genotypic characterisation methods to demonstrate relationships among the outbreaks.

## Materials and Methods

### *Definitions*

For the purpose of this study, a *case* was defined as a turkey showing the clinical signs of fowl cholera from which an isolate of *P multocida* was obtained. An *outbreak* was defined as the occurrence of clinical fowl cholera in two or more turkeys within a flock on a single farm.

### *Bacteria*

The 22 field isolates of *P multocida* examined in this study are listed in Table 1. Reference strains for the 3 subspecies of *P multocida* subsp *gallicida, multocida* and *septica* were obtained from Dr R Mutters (Department of Medical Microbiology, Marburg, Germany). These reference strains were NCTC10204 (subsp *gallicida*), NCTC10322 (subsp *multocida*) and CIP A125 (subsp *septica*).

### *Field Information*

The 7 farms involved in this study form two geographical groups. Farms 1, 2, 4 and 6 are located near Camden. Farm 1 is about 70 km south-west of Camden. Farms 2, 4 and 6 are located 10 km north of Camden and are within 5 km of each other. Farms 3, 5 and 7, the second geographical group, are located in the Tamworth district. These farms are located between 80 to 100 km south or south-west of Tamworth and are separated by at least 30 km. All 7 farms were operated on an 'all in - all out' basis except farm 5, which was a multi-age complex. All farms were producing meat turkeys and all obtained their birds from the one breeding company. On the basis of the available field evidence, the 16 cases of fowl cholera studied were grouped into 8 different outbreaks, numbered I to VIII. With the

TABLE 1
**Origin of isolates used in this study**

| Farm | Outbreak | Date* | Study code | Source |
|---|---|---|---|---|
| 1 | I | 10/92 | PM137 | Lung Bird 1 |
| | II | 2/93 | PM1 | Lung Bird 1 |
| | | 3/93 | PM12 | Lung Bird 2 |
| | | 3/93 | PM13 | Lung Bird 3 |
| | | 3/93 | PM14 | Lung Bird 4 |
| 2 | III | 2/93 | PM2 | Heart Bird 3 |
| | | 2/93 | PM3 | Heart Bird 3 |
| | | 2/93 | PM4 | Heart Bird 3 |
| | | 2/93 | PM5 | Lung Bird 3 |
| | | 2/93 | PM6 | Lung Bird 2 |
| | | 2/93 | PM7 | Lung Bird 2 |
| | | 2/93 | PM8 | Lung Bird 2 |
| | | 2/93 | PM9 | Lung Bird 1 |
| | | 2/93 | PM10 | Heart Bird 2 |
| | | 2/93 | PM11 | Lung Bird 4 |
| | | 3/93 | PM15 | Lung Bird 5 |
| | | 3/93 | PM16 | Lung Bird 6 |
| 3 | IV | 10/92 | PM132 | Lung Bird 1 |
| 4 | V | 10/92 | PM133 | Lung Bird 1 |
| 5 | VI | 10/92 | PM136 | Lung Bird 1 |
| 6 | VII | 12/92 | PM135 | Lung Bird 1 |
| 7 | VIII | 12/92 | PM134 | Lung Bird 1 |

* Date given as month/year

TABLE 2
**Overall field information**

| Farm | Outbreak | Date | No. of sheds affected | No. of birds in affected sheds* | % of birds affected† |
|---|---|---|---|---|---|
| 1 | I | 10/92 | 2 | 14 500 | 16 and 6 |
| | II | 2/93 and 3/93 | 3 | 11 700 | 5, 0.5 and 6 |
| 2 | III | 2/93 and 3/93 | 2 | 12 600 | 3 and 9 |
| 3 | IV | 10/92 | 2 | 20 600 | 23 (combined) |
| 4 | V | 10/92 | 2 | 12 600 | 3 and 3 |
| 5 | VI | 10/92 | 3 | 19 000 | 25 (combined) |
| 6 | VII | 12/92 | 1 | 3 200 | 1 |
| 7 | VIII | 12/92 | 2 | 14 500 | 3.5 (combined) |

* Total numbers in all affected sheds are presented

† The percentage of affected birds in each shed is presented except for those farms where only a combined figure for all affected sheds was only available

exception of farm 1, each farm represented a different outbreak. That the 2 outbreaks on farm 1 occurred in separate flocks, combined with an absence of clinical cases in the intervening period, meant that these outbreaks were regarded as distinct and different outbreaks. The available field information on the outbreaks is presented in Table 2.

### *Phenotypic Characterisation*

All field isolates and reference strains were subjected to a full phenotypic characterisation. The Gram stain reaction of the organisms was determined using the procedure of Burke as described by Cruickshank *et al* (1975). The presence of catalase, oxidase, ß-galactosidase and urease activity and the ability to produce indole were determined as described by Cowan (1974). The ability to ferment the following carbohydrates was tested using a microplate fermentation method: L-arabinose, dulcitol, D-glucose, D-lactose, maltose, D-mannitol, D-sorbitol, D-sucrose, D-trehalose and D-xylose. The microfermentation method used a basal medium (1% (w/v) peptone, 0.5% (w/v) NaCl, 0.005% (w/v) bromocresol purple) adjusted to pH 6.8. The basal medium was autoclaved and then aseptically supplemented with sterile carbohydrate to a final concentration of 1% (w/v). The complete medium was dispensed in 200 µL volumes into sterile tissue culture quality microtitre plates. Heavy suspensions of the organisms (1 loopful in 2 mL nutrient broth) were prepared and 10 µL added to each carbohydrate well. The plates were incubated at 37°C and read daily for 3 days.

### *Genotypic Characterisation*

REA was performed on all the field isolates. Chromosomal DNA was extracted by a scaled-down version of standard DNA extraction methods. Briefly, the bacteria were grown overnight in 150 mL of brain heart infusion broth[§] and the bacterial cells collected by centrifugation and washed 3 times in phosphate buffered saline (PBS) pH 7.2. The cells were resuspended in 1 mL of saline-EDTA (0.85% saline, 0.05M EDTA), 1 mL of lysozyme (20 mg/mL) added and the suspension held at 37°C for 1 h. Next, 10 µL of 25% sodium dodecyl sulphate and 100 µL of Proteinase K (2 mg/mL) were added and the suspension incubated at 60°C for 1 h. 10.5 µL of RNAase (10 mg/mL) was added and the mixture held at 37°C for 30 min. The suspension was then subjected to repeated phenol-chloroform extractions. The final supernatant was supplemented with 0.25 volumes of 3 M sodium acetate and 2.5 volumes of ice-cold ethanol, held at -70°C for 1 h and centrifuged (14 000 *g*, 20 min). The DNA pellet was then resuspended in distilled water and the concentration of the DNA determined by spectrophotometric methods. The DNA samples were subjected to restriction digestion using the restriction enzyme *Hpa*II. The digests were performed by a standard method (Sambrook *et al* 1989). Electrophoresis of the resultant digest was conducted in a 0.7% agarose gel using TPE buffer (0.08M Tris-phosphate, 0.002M EDTA) at 20V for 16 to 17 h. The gels were photographed using ultraviolet light and Kodak Technical Pan film (Morrow and Olcayoz 1989).

§ Oxoid CM225, Oxoid Australia, West Heidelberg, Vic

With some exceptions, ribotyping was performed as described previously (Snipes *et al* 1990a). Briefly, the restriction digests used for REA were subjected to agarose gel electrophoresis and then transferred to a nylon membrane using a vacuum blotting system. The membrane was hybridised with a probe known as the pHpg probe using standard methods (Sambrook *et al* 1989). The pHpg probe consisted of the plasmid pUC19 into which the 16S rRNA of *H paragallinarum* had been inserted. Before use, the probe was converted to a linear form by digestion with the restriction enzyme *Pst*I and then labelled with digoxygenin as described by the manufacturer (Boehringer Mannheim, Mannheim, Germany). The presence of probe on the membrane was determined using the immunological detection method, as detailed by the manufacturer.

## Results

### *Phenotypic Characterisation*

All 22 field isolates were gram negative rods that produced indole, were oxidase and catalase positive and ß-galactosidase and urease negative and fermented glucose, mannitol and sucrose but not dulcitol or lactose. All the isolates were identified as *P multocida.* The field isolates differed in their ability to ferment maltose, sorbitol, trehalose and xylose. These differences allowed the recognition of 4 distinct biochemical types, termed biovars A, B, C and D. Biochemical biovars A, B and C matched the described properties of *P multocida* subsp *multocida* (Mutters *et al* 1985), with biovar A, the most common biovar, giving exactly the same pattern of reactions as

TABLE 3
**Biochemical differention of Australian isolates and reference strains of *P multocida***

| Acid produced from | subsp *multocida* | | | | subsp *septica* | | subsp *gallicida* |
|---|---|---|---|---|---|---|---|
| | Reference strain | Biovar A* | Biovar B† | Biovar C‡ | Reference Strain | Biovar D§ | Reference Strain |
| Arabinose | - | - | - | - | - | - | + |
| Maltose | - | - | - | - | - | + | - |
| Sorbitol | + | + | + | + | - | - | + |
| Trehalose | - | - | - | + | + | + | - |
| Xylose | + | + | - | + | + | + | - |

* Biovar A isolates, PM2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 15, 16, 132, 133, 136 and 137.
† Biovar B isolate, PM134.
‡ Biovar C isolates, PM1, 12, 13 and 14.
§ Biovar D isolate, PM135.

the reference strain of *P multocida* subsp *multocida*. The other field isolate formed biovar D. As this isolate, with the exception of maltose fermentation, gave the same pattern of reactions as the reference strain of *P multocida* subsp *septica* (Mutters *et al* 1985), it was identified as belonging to subsp *septica*. The differential properties of the field isolates and the reference strains are listed in Table 3.

### *Genotypic Characterisation*

REA using *Hpa*II generated useful 'fingerprint' patterns that allowed the assignment of the 22 isolates to 7 different REA patterns. Figure 1 illustrates the 7 different REA profiles.

Ribotyping allowed the recognition of 7 different ribotype patterns, illustrated in Figure 2. The 7 groups recognised by ribotyping matched the groups established by REA.

### *Overall Results*

The overall phenotypic and genetic characterisation results are presented in Table 4. All 3 typing methods indicated that outbreaks I and II on farm 1 were distinct outbreaks caused by 2 different organisms. The multiple isolates obtained from outbreak II were all the same biovar and all gave identical REA and ribotyping profiles. All three typing methods indicated that all isolates from outbreak III on farm 2 were identical, suggesting that only one organism was involved in this outbreak. The molecular methods indicated that outbreaks II, III, IV, VI, VII and VIII were all caused by distinct and different organisms, but outbreaks I and V were caused by organisms with the same characteristics. Biotyping was less discriminatory and suggested that outbreaks I, II, VII and VIII were caused by different organisms but could not separate outbreaks I, III, IV, V and VI.

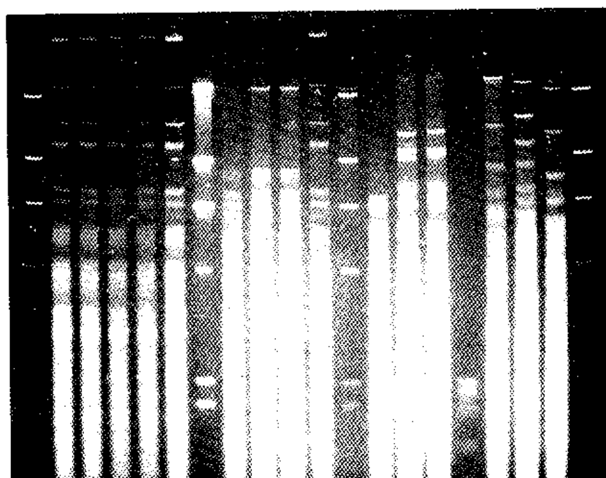


Figure 1. *Hpa*II REA profiles of representative isolates. Lanes are numbered from the left and are as follows: 1 Molecular weight marker, 2 PM2, 3 PM3, 4 PM5, 5 PM6, 6 PM9, 7 Molecular weight marker, 8 PM1, 9 PM12, 10 PM13, 11 PM15, 12 Molecular weight marker, 13 PM132, 14 PM133, 15 PM137, 16 Lambda DNA cut by *Hpa*II, 17 PM136, 18 PM135, 19 PM134, 20 Molecular weight marker. The REA profiles are as follows: I lanes 14 and 15; II lane 13; III lane 17; IV lane 18; V lane 19; VI lanes 8, 9, 10; VII lanes 2, 3, 4, 5, 6, 11.

## Discussion

Our characterisation study established that 21 of the 22 isolates of *P multocida* belonged to the subspecies *multocida*. This is consistent with findings in overseas studies of fowl cholera in turkeys. Snipes *et al* (1990b) found that subspecies *multocida* accounted for 95.5% of 333 isolates of *P multocida* from turkeys dead of fowl cholera and for 98% of 88 isolates of *P multocida* from live turkeys.

According to Ogle *et al* (1987), an epidemiological marker should: 1) be sufficiently sensitive to distinguish all unrelated isolates,

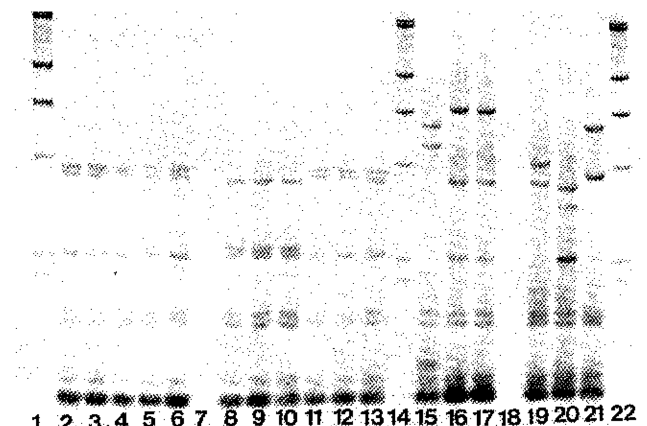


Figure 2. Ribotyping profiles of representative isolates. Lanes are as follows: 1 Molecular weight marker, 2 PM2, 3 PM3, 4 PM5, 5 PM6, 6 PM9, 7 Blank, 8 PM1, 9 PM12, 10 PM13, 11 PM11, 12 PM15, 13 PM16, 14 Molecular weight marker, 15 PM132, 16 PM133, 17 PM137, 18 Blank, 19 PM136, 20 PM135, 21 PM134, 22 Molecular weight marker. The ribotypes are as follows: i lanes 16 and 17; ii lane 15; iii lane 19; iv lane 20; v lane 21; vi lanes 8, 9, 10; vii lanes 2, 3, 4, 5, 6, 11, 12, 13.

TABLE 4
**Summary of characterisation of *P multocida* isolates using biochemical and molecular methods**

| Farm | Outbreak | Isolates | Date | Subspecies | Biovar | REA type | Ribo-type |
|---|---|---|---|---|---|---|---|
| 1 | I | PM137 | 10/92 | *multocida* | A | I | i |
| | II | PM1 | 2/93 | *multocida* | C | VI | vii |
| | | PM12,13,14 | 3/93 | *multocida* | C | VI | vi |
| 2 | III | PM2,3,4,5,6, 7,8,9,10,11 | 2/93 | *multocida* | A | VII | vii |
| | | PM15,16 | 3/93 | *multocida* | A | VII | vii |
| 3 | IV | PM132 | 10/92 | *multocida* | A | II | ii |
| 4 | V | PM133 | 10/92 | *multocida* | A | I | i |
| 5 | VI | PM136 | 10/92 | *multocida* | A | III | iii |
| 6 | VII | PM135 | 12/92 | *septica* | D | IV | iv |
| 7 | VIII | PM134 | 12/92 | *multocida* | B | V | v |

2) specifically identify all related isolates, and 3) be stable. We have used these same criteria – sensitivity, specificity and stability – to evaluate the typing methods used in this study. It should be recognised that our data on stability are limited by the fact that multiple isolates were only available from 2 of the 8 outbreaks studied.

REA and ribotyping profiles proved to be specific, sensitive and stable. Both techniques were able to clearly separate outbreaks II, III, IV, VI, VII and VIII. Both techniques indicated that all isolates within outbreak III were the same type. Thus, the techniques were specific and sensitive. As well, both techniques indicated that all 4 isolates from outbreak II, which were derived from four different birds, were the same type. In outbreak III, the 12 isolates examined were collected from 6 birds with multiple isolates being obtained from 2 birds. Thus, our work has established that REA and ribotyping profiles are stable *in vivo*. Our results for outbreaks II and III also suggest that a clinical outbreak of fowl cholera is associated with a single REA or ribotype profile. Our finding that both REA and ribotyping are useful techniques for studies on fowl cholera outbreaks has also been reached by others (Kim and Nagaraja 1990; Snipes *et al* 1990a; Carpenter *et al* 1991; Christiansen *et al* 1992a, b).

Biotyping provided only limited sensitivity and specificity. As only 4 biovars were recognised, outbreaks I, III, IV, V and VI could not be separated. In contrast, REA and ribotyping profiles clearly demonstrated that outbreaks III, IV and VI were unrelated while outbreaks I and V were related.

The biovar shift seen between the two outbreaks on farm 1 was supported by a shift in the REA and ribotype profiles. Hence, we conclude that the 2 outbreaks seen on farm 1 were caused by distinctly different organisms.

Serological characterisation of isolates was not undertaken although somatic and capsular types have been used in the past for epidemiological studies.

The 1992 outbreaks of fowl cholera occurred in two geographical locations. Farms 1, 4 and 6 were located close to Camden while farms 3, 5 and 7 were located near Tamworth. The proximity of the farms in the 2 groups and of the outbreaks made it difficult on the basis of the field data to decide whether or not the outbreaks were related. The results of this study support speculation that outbreaks I and V on farms 1 and 4 were related. As well, the results clearly indicate that all the other 1992 outbreaks were unrelated.

The retrospective nature of this study meant that only 1 isolate was available for study from outbreaks IV, V, VI, VII and VIII. Hence it is not possible to confidently rule out the possibility that more than 1 organism might have been present in these outbreaks.

A series of similar studies have been performed on fowl cholera outbreaks in turkey flocks in California (Carpenter *et al* 1991; Christiansen *et al* 1992a, b). In general our findings mirror those reported by the Californian group. In both California and Australia, a diverse range of REA and/or ribotypes have been associated with fowl cholera outbreaks on different farms. In the detailed study of 3 farms performed by Christiansen *et al* (1992b), multiple samplings demonstrated the presence of only a single REA type in 2 of the 3 farms with the third farm showing a range of different types. This occurrence of a single REA type matches our findings on farm 2.

The confirmation that a diverse collection of *P multocida* isolates was responsible for the fowl cholera outbreaks was achieved only by the use of the molecular methods. Initially, the proximity of the farms and the outbreaks led to suspicions that the outbreaks were caused by the same strain. A conclusion that can be drawn from our finding that a range of organisms were involved is that some other common precipitating factor, such as stress, might have been important. There is a need for further studies into the epidemiology of fowl cholera outbreaks. The use of molecular typing techniques such as those used in this study, will assist in understanding the epidemiology of this disease, particularly in studies of factors which precipitate outbreaks.

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