MOLECULAR IDENTIFICATION AND EPIDEMIOLOGY

Demonstration that Australian *Pasteurella multocida* isolates from sporadic outbreaks of porcine pneumonia are non-toxigenic (*toxA*[−]) and display heterogeneous DNA restriction endonuclease profiles compared with toxigenic isolates from herds with progressive atrophic rhinitis

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Capsular types A and D of *Pasteurella multocida* cause economic losses in swine because of their association with progressive atrophic rhinitis (PAR) and enzootic pneumonia. There have been no studies comparing whole-cell DNA profiles of isolates associated with these two porcine respiratory diseases. Twenty-two isolates of *P. multocida* from diseased pigs in different geographic localities within Australia were characterised genotypically by restriction endonuclease analysis (REA) with the enzyme *Cfo*I. Seven of 12 *P. multocida* isolates from nasal swabs from pigs in herds where PAR was either present or suspected displayed a capsular type D phenotype. These were shown to possess the *toxA* gene by polymerase chain reaction (PCR) and Southern hybridisation, and further substantiated by production of cytotoxin *in vitro*. The *Cfo*I profile of one of these seven isolates, which was from the initial outbreak of PAR in Australia (in Western Australia, WA), was identical with profiles of all six other toxigenic isolates from sporadic episodes in New South Wales (NSW). The evidence suggests that the strain involved in the initial outbreak was responsible for the spread of PAR to the eastern states of Australia. Another 10 isolates, representing both capsular types A and D, were isolated exclusively from porcine lung lesions after sporadic outbreaks of enzootic pneumonia in NSW and WA. *Cfo*I restriction endonuclease profiles of these isolates revealed considerable genomic heterogeneity. Furthermore, none of these possessed the *toxA* gene. This suggests that *P. multocida* strains with the *toxA* gene do not have a competitive survival advantage in the lower respiratory tract or that toxin production does not play a role in the pathology of pneumonic lesions, or both. REA with polyacrylamide gel electrophoresis and silver staining was found to be a practical and discriminatory tool for epidemiological tracing of *P. multocida* outbreaks associated with PAR or pneumonia in pigs.

Introduction

Pasteurella multocida is implicated as an aetiological agent in progressive atrophic rhinitis (PAR) in pigs and is commonly isolated from the lungs of pigs with enzootic pneumonia. The progressive degeneration of nasal turbinate tissue seen in PAR is predominantly associated with toxigenic strains of *P. multocida* [1–3].

Hybridisation studies have demonstrated that toxigenic strains of *P. multocida* (of capsular types A and D) contain a chromosomally located *toxA* gene, whilst non-toxigenic strains do not [4]. Toxigenic strains reproduce the clinical symptoms of PAR when experimentally inoculated into pigs [5] and express a 145-kDa dermonecrotic toxin (DNT). This toxin is lethal for mice [1], induces osteolysis in swine turbinate bones and produces haemorrhagic and necrotic lesions in guinea-pig skin [6]. Toxigenic strains demonstrate *in vitro* toxigenicity for tissue culture cell lines, including embryonic lung and Vero cells, and such assays are used

as diagnostic tools [7, 8]. Furthermore, a recombinant toxin derivative with a deletion of 121 amino acids in the N-terminal region of the native DNT [9] has been shown to be non-toxigenic and protective as a vaccine against PAR in gilts challenged intranasally with *Bordetella bronchiseptica* and *P. multocida* [10].

Five capsular types (A, B, C, D and E) are recognised within the genus *Pasteurella*, with types A and D most frequently isolated from the respiratory tract of pigs [11]. Although both toxigenic and non-toxigenic varieties of capsular types A and D have been described, toxigenic isolates from pigs with PAR in Australia are almost exclusively capsular type D [2] whilst *P. multocida* isolates from pneumonic lesions are typically non-toxigenic, capsular type A [3, 12–15]. Two field surveys conducted in New South Wales (NSW), Australia, during 1989–1990 revealed that lung isolates of *P. multocida* are widespread across different herds with a strong association between lesion severity and frequency of isolation of the organism [3]. Non-toxigenic, capsular type A isolates of *P. multocida* have also been associated with severe pleuritis in two North American herds, where grower and finisher pigs showed severe emaciation and a high mortality rate [13].

Toxigenic *P. multocida* cannot be reliably differentiated biochemically or by colony morphology from non-toxigenic varieties. Thus, various toxin detection assays including cell culture [2, 16], ELISA [17], immunoblotting [18], dot-blot hybridisations [4] and PCR [18–20] have been developed and evaluated. Restriction endonuclease analysis studies have been applied to compare whole-cell DNA fingerprints of isolates of *P. multocida* from herds with clinical signs of PAR [21, 22]. However, there are few reports addressing genetic heterogeneity among pneumonic isolates of *P. multocida* or a comparison of whole-cell DNA restriction endonuclease profiles between strains of *P. multocida* causing pneumonia and PAR. In this study whole-cell DNA was purified from 22 isolates of *P. multocida* and digested with *Cfo*I. Restriction endonuclease profiles, resolved by polyacrylamide gel electrophoresis and visualised by silver staining, were compared. Twelve of the 22 isolates were from the nasal passages of pigs with clinical symptoms of PAR whilst the remaining 10 isolates were from pneumonic lung lesions during sporadic outbreaks of pneumonia in geographically diverse Australian herds. The capsular type of these strains was determined and the presence of the *tox*A gene was confirmed by Southern hybridisation and PCR. In addition, toxin production in each of these isolates was assessed by a cytotoxin assay.

Materials and methods

Culture of *P. multocida*

Strains of *P. multocida* used in this study are listed in Table 1. The geographic location of all 22 isolates is

depicted in Fig. 1. *P. multocida* isolates 7, 10 and 11 were from pneumonic lung lesions from pigs and were stored as freeze-dried ampoules during 1969–1970. Records identifying the sources of these strains are no longer available. However, these isolates were cultured from lungs by the Regional Veterinary Laboratory at Glenfield which serviced the Hunter, central and far west, southern tablelands and south coast of NSW as well as the metropolitan region of Sydney. All lung isolates were from samples obtained from pigs at necropsy and cultured on blood agar (sterile sheep blood agar 10% v/v; Columbia agar base 3.85% w/v; granulated agar 0.73% w/v). Twelve isolates were from nasal swabs of the turbinates of pigs with clinical signs of PAR cultured on selective blood agar [2]. From lyophilised stocks of these isolates, subcultures were grown on blood agar incubated overnight at 37°C in CO₂ 5% v/v in air. From these plates, brain heart infusion broth (BHIB) 3.7% was inoculated with a single colony of *P. multocida* and shaken (200 oscillations/min) for 4–6 h until mid-log phase. *P. multocida* was harvested by centrifugation (10 000 rpm, 15 min; Sorvall SS34 head) and washed twice with sterile TBS (10 mM Tris-HCl, pH 7.4; NaCl 0.9%).

Phenotypic characteristics

All *P. multocida* isolates were stained by Gram's method [23]. The identity of bacterial isolates was confirmed by standard biochemical tests [24].

Capsular typing of *P. multocida* isolates

P. multocida isolates were classed as capsular type A or D by the acriflavine and hyaluronidase test, respectively [2]. Briefly, one drop of freshly prepared acriflavine solution (diluted 1 in 1000 with milliQ water; Sigma) was mixed with a colony of bacteria taken from an overnight blood-agar culture. Type D bacteria, but not type A, produce a coarse flocculent precipitate within 30 min at room temperature. The hyaluronidase test was performed by first impregnating 5 × 30 mm sterile Whatman filter strips in a sterile solution of ovine testicular hyaluronidase (700 U/ml; Sigma). These strips were then laid across blood agar plates pre-streaked with each *P. multocida* isolate. After incubation at 37°C in CO₂ 5%, the continuity of growth was assessed. Growth of type A but not type D cultures was inhibited in the immediate vicinity of the hyaluronidase strip.

Toxin tests

A cellular cytotoxicity assay [2] was used to determine the production of toxins by isolates of *P. multocida*. Briefly, bovine turbinate cells (BTC) were cultured in 0.5 ml of basal medium Eagle (modified) with Hanks's salt (BME/H) containing fetal calf serum (FCS) 10% v/v, penicillin 100 U/ml and streptomycin 100 U/ml. BTC had a passage level of 34. Each well of a 24-well plate (Linbro, Flow Laboratories) was seeded with

Fig. 1. Map of Australia identifying the geographic location of *P. multocida* isolates used in this study.

1.6×10^5 cells, incubated at 37°C with CO_2 5% in air for 5 days at which time confluency had been reached. The cells were washed once with phosphate-buffered saline (PBS: pH 7.4, free of calcium and magnesium ions) and the cells in each well were overlaid with 0.5 ml of antibiotic-free BME/H containing FCS 4% v/v and agarose (Seaplaque, Marine Colloids) 2% w/v. The agarose top layer was allowed to solidify at room temperature for 10 min before each *P. multocida* isolate was inoculated on to half of the agarose surface of each well. A toxigenic strain of *P. multocida* was always included in each test run. Plates were incubated at 37°C and CO_2 5% in air for 72 h before observation. The presence of toxin was confirmed by observing the morphology of BTC through an inverted microscope ($25\times$ magnification). Typically, cytotoxin-producing strains induced BTC to appear more granular and rounded compared with non-toxigenic isolates of *P. multocida* [2]. Isolates were tested in triplicate wells for each treatment and examined for cytotoxicity after incubation for 18, 24, 48 and 72 h.

Extraction of genomic DNA

P. multocida isolates were grown overnight on blood agar (3×90 mm plates/isolate) which were flooded

with 5 ml of sterile PBS and cells were gently removed by scraping with a glass L-shaped rod. The resuspended bacteria were pelleted by centrifugation and washed three times with PBS. DNA was extracted essentially as described previously [25, 26]. Briefly, the bacterial pellets were resuspended in 4 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.4) containing sucrose 10%. Lysozyme (50 mg) was added and the mixture was incubated at room temperature (RT) for 10 min before the addition of 50 μl of SDS 10% and 30 μl of RNAase A (10 mg/ml), then incubated at 37°C for 1 h before proteinase K (500 μg) was added. The resultant mixture was incubated at 37°C for 2 h followed by incubation at 56°C overnight. This mixture was then extracted three times with phenol:chloroform: isoamyl alcohol (25:24:1) and once with chloroform: isoamyl alcohol in serum separator tubes (Becton Dickinson). Finally, the aqueous phase containing the DNA was dialysed overnight with three changes of TES buffer (TES: 10 mM Tris HCl, pH 7.4, 1 mM EDTA, 100 mM NaCl). The concentration and purity of DNA were determined spectrophotometrically. Although large amounts of DNA (3–5 mg) were readily recovered by the above protocol, this study was unable to reliably generate clear *Cfo*I restriction profiles with DNA recovered from toxigenic, capsular type D

isolates. An alternate DNA extraction protocol based on hexadecyltrimethylammonium bromide (CTAB) was used to extract whole-cell DNA from these isolates. Briefly, 200 mg of bacterial pellet were resuspended in 2 ml of TSK (TSK: 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, SDS 0.5% and proteinase K 200 µg) and incubated at 37°C for 1 h. A 280-µl volume of 5 M NaCl was added followed by 200 µl of CTAB solution (CTAB: hexadecyltrimethylammonium bromide 10% w/v in 0.3 M NaCl). After mixing by inversion, the DNA lysate was incubated for 10 min at 65°C. DNA was purified by phenol:chloroform:isoamyl alcohol extraction as described above.

Southern hybridisation

Plasmid *pPMF3.5* (kindly supplied by Dr E. M. Kamp, Department of Bacteriology, Central Veterinary Institute, Lelystad, The Netherlands) was transformed into *E. coli* strain DH5α, cultured on LB medium containing carbenicillin 50 µg/ml overnight with shaking and pelleted by centrifugation (5000 rpm, SS34 head). Plasmid DNA was extracted by column chromatography according to the manufacturer's instructions (Qiagen, Hilden, Germany). *pPMF3.5* which contains the 3' half of the *toxA* gene was mapped with the restriction endonucleases *EcoRI*, *XbaI*, *HindIII* and combinations thereof to confirm the identity of the plasmid construct [4]. *pPMF3.5* and lambda DNA digested with *HindIII* were labelled with ³²P with an Amersham random priming kit (Amersham). *P. multocida* genomic DNA digested with *CfoI* was resolved in agarose 1.0% gels, transferred to nylon membrane, cross-linked with ultraviolet light and hybridised with radiolabelled *pPMF3.5* [27]. The nylon membrane was washed three times under stringent temperature and salt conditions (65°C, 2 × SSC) and exposed to film (Fuji RX, Japan) at -80°C for 1-3 days.

Polyacrylamide gel electrophoresis

P. multocida whole-cell DNA (1-2 µg) digested with the restriction endonuclease *CfoI* was loaded on to polyacrylamide 3.5% gels (12 × 16 cm) and immediately pulsed into the gel matrix at 80 V for 10 min. The gels were electrophoresed overnight (17-19 h, 35 V/gel) in TBE buffer (TBE: 90 mM Tris-HCl, 90 mM borate, 2 mM EDTA) with a Hoefer maxi gel apparatus as described previously [25]. The gels were removed, washed once with milliQ water and stained with silver as described by the manufacturer (Rapid Silver Stain, INC Biomedical). Gels were fixed in acetic acid 0.1% and photographed immediately.

Polymerase chain reaction

Primers which amplify a 1.2-kb fragment of the *toxA* gene of *P. multocida* [18] were used to confirm the presence of the *toxA* gene. Primer sequences were as follows: primer 1, 5'-TACTCAATTGAAAAGCGC

TTTATCTTCC-3' and primer 2, 5'-TCTACTACAGTTGCTGGTATTTTAAATAT-3'. PCR amplification conditions were similar to those described by Nagai *et al.* [18] and consisted of the following: 1 µM of each oligonucleotide primer, 200 µM of each nucleotide triphosphate, 2.5 U of Taq polymerase (Stoffle enzyme), 1 × reaction buffer (10 mM Tris-HCl, pH 8.3, 10 mM KCl) and sample DNA (100 ng) in a final volume of 50 µl. The reaction mixture was subjected to an amplification regimen consisting of an initial denaturation step (95°C, 2 min) followed by 25 cycles of 92°C (1 min), 55°C (1 min) and 70°C (2 min) with a Corbett Research FTS 960 thermocycler. A portion of the reaction mixture (15 µl) was electrophoresed through agarose 1.0% gel with 0.5 × TBE buffer, 60 V for 1.5 h. The PCR amplification product was visualised with ethidium bromide and photographed with Polaroid 667 film.

Results

Capsular typing of porcine *P. multocida* isolates

P. multocida isolates 1, 3, 4, 7, 8, 9, 10 and 14-21 produced a type D capsule; all other isolates (2, 5, 6, 11, 12, 13 and 22) were capsular type A as defined by the hyaluronidase and acriflavine tests (Table 1).

Evidence of the presence and expression of the *toxA* gene

Table 1 shows that all 10 *P. multocida* isolates from pneumonic lesions did not secrete cytotoxic toxin in an agarose overlay bovine turbinate cell assay. In contrast, *P. multocida* isolates 1, 15, 16, 17, 18, 19, 20 and 21 (capsular type D) which had been isolated from the nasal turbines of pigs in a herd with symptoms of PAR, induced cytotoxic changes to bovine turbinate cells (Table 1). A single capsular type A isolate (isolate 22 from Cootamundra, NSW, Australia) also demonstrated cytotoxin activity. Isolate 2, which was co-isolated with isolate 1 from the same nasal swab, did not express toxin. Similarly, isolate 3, from the same farm (Byford, Western Australia, Australia), also failed to express toxin. Isolate 8 (Bendigo, Victoria, Australia), from a nasal swab from a pig with nasal discharge but with no evidence of turbinate atrophy, failed to demonstrate cytotoxin activity.

The presence of the *toxA* gene amongst the 22 isolates of *P. multocida* was examined by Southern hybridisation and PCR analyses of purified DNA. Southern blotting of the *CfoI*-digested whole-cell DNA from *P. multocida* when probed with ³²P-labelled *pPmF3.5*, confirmed that the 10 pneumonic isolates did not possess the *toxA* gene. Nasal swab isolates 2, 3 and 8 also did not hybridise with ³²P-labelled *pPmF3.5*. However, *CfoI*-digested whole-cell DNA from toxigenic isolates 15-21 showed the presence of three strongly hybridising fragments of *c.* 3.9, 1.9 and a

Table 1. Geographic location, capsular type and toxigenic status of *P. multocida* isolates

Isolate no.	Origin and identity	Type	Syndrome	Source	Cytotoxicity	<i>toxA</i> PCR	<i>toxA</i> probe
1	WA 1096-849	D	PAR	N. swab	+	+	ND
2	WA 1097-852	A	PAR	N. swab	—	—	—
3	WA 1098-855	D	PAR	N. swab	—	—	—
12	WA 1397	A	Pneumonia	Lung	—	—	—
13	WA 1456	A	Pneumonia	Lung	—	—	—
4	WN 2187	D	Pneumonia	Lung	—	—	—
5	WN 2214-3	A	Pneumonia	Lung	—	—	—
6	WN 2214-4	A	Pneumonia	Lung	—	—	—
7	EMAI 221	D	Pneumonia	Lung	—	—	—
8	EMAI 1697	D	N. discharge	N. swab	—	—	ND
9	EMAI 234	D	Pneumonia	Lung	—	—	—
10	EMAI 235	D	Pneumonia	Lung	—	—	—
11	EMAI 241	A	Pneumonia	Lung	—	—	—
14	EMAI 199	D	Pneumonia	Lung	—	—	—
15	EMAI 1155	D	PAR	N. swab	+	+	+
16	EMAI 1970	D	PAR	N. swab	+	+	+
17	EMAI 1279	D	PAR	N. swab	+	+	+
18	EMAI 1295	D	PAR	N. swab	+	+	+
19	EMAI 1325	D	PAR	N. swab	+	+	+
20	EMAI 1376	D	PAR	N. swab	+	+	+
21	EMAI 1656	D	PAR	N. swab	+	+	+
22	EMAI 2256	A	PAR	N. swab	+	+	ND

PAR, progressive atrophic rhinitis; N. swab, nasal swab; N. discharge, nasal discharge; ND, not done.

fragment of <0.56 kb, thereby confirming the presence of the *toxA* gene (data not shown). These fragment sizes were in agreement with predicted values based on the presence of *CfoI* restriction sites identified in the DNA sequence of the *toxA* gene (accession no. X52478).

PCR primers [18] facilitated the amplification of a 1.2-kb fragment of the *toxA* gene from whole-cell DNA purified from isolates 1 and 15–22. DNA from isolates of *P. multocida* which were not toxigenic in the agarose gel overlay assay (isolates 2–14) and which did not hybridise with *pPmF3.5* (data not shown) also failed to show any amplification product in the PCR (Table 1).

Genomic characterisation

Isolates from nasal swabs. Isolates 15–21 were obtained during sporadic outbreaks of progressive atrophic rhinitis in pig herds at different geographic localities in NSW (Fig. 2a). DNA from these seven isolates displayed remarkably similar *CfoI* restriction endonuclease profiles (Fig. 2a). Apart from a single, darkly stained fragment with a molecular size of *c.* 4.6 kb (arrowhead, lane 5), the *CfoI*-generated restriction endonuclease profiles of these seven isolates were indistinguishable. The *CfoI* DNA profile of isolate 8 (non-toxigenic, capsular type D) was clearly different from the typical *CfoI* profile of toxigenic capsular type D isolates (Fig. 2b). Toxigenic isolate 1 (Byford, WA) was amongst the first toxigenic isolates detected in Australia soon after the appearance of PAR [8] and approximately 2 years before the appearance of PAR in herds in NSW (isolates 15–21). The *CfoI* restriction endonuclease profile of isolate 1 was remarkably similar to the *CfoI* profiles of NSW toxigenic isolates 15–21 (Fig. 3).

P. multocida isolates 1, 2 and 3 were from nasal swabs collected from one herd located in Byford, WA. The *CfoI* profiles of these three isolates were different (Figs. 3 and 4). The results for isolates 1, 2 and 3 clearly show that isolates of *P. multocida* with different capsular types, genotypes and toxigenic status may co-exist in the same animal or within the same herd.

Toxigenic, capsular type A isolate. Toxigenic, capsular type A isolates of *P. multocida* are rarely cultured from swine. DNA from capsular type A, toxigenic isolate 22, displayed a distinctly different *CfoI* profile compared with the other 21 isolates (Fig. 3).

Pneumonic isolates. Restriction endonuclease analysis (with *CfoI*) of DNA purified from whole-cell lysates of pneumonic *P. multocida* isolates 4, 5, 6, 7, 9, 10, 11, 12, 13 and 14, irrespective of capsular type, revealed considerable heterogeneity (Figs. 3 and 4). Isolates 4, 5 and 6, all from pigs in herds located near Murwillumbah, NSW, generated readily discernible *CfoI* profiles compared with one another and with all other isolates (Figs. 3 and 4). *CfoI* profiles of DNA from isolates 7 and 10 were virtually indistinguishable from one another (except for a single fragment *c.* 6.7 kb in size, identified with an arrowhead), indicating a close genetic relationship (Fig. 4, lanes 5 and 6). *CfoI* profiles of DNA from isolates 9 (Condobolin, NSW) and 11 were readily discernible from one another and from all other isolates. DNA from isolate 14 (Joadja, NSW) also generated a unique *CfoI* profile (Fig. 4).

Isolates 5 (Murwillumbah, NSW) and 11, both capsular type A, non-toxigenic isolates, displayed similar *CfoI* DNA profiles, although each could be easily distinguished from the other by the presence of high mol. wt fragments (Fig. 3). Capsular type A isolates

Fig. 2. Silver-stained polyacrylamide 3.5% gel showing *CfoI* restriction endonuclease profiles of whole-cell DNA purified from toxigenic isolates of *P. multocida*. **A:** Lane 1, isolate 19 (Deniliquin, NSW); 2, isolate 15 (Canowindra, NSW); 3, isolate 18 (Galore, NSW); 4, isolate 17 (Henty, NSW); 5, isolate 21 (Tyringham, NSW); 6, isolate 20 (Molong, NSW); 7, isolate 16 (Hillston, NSW); 8, lambda DNA digested with *HindIII*. **B:** Lane 1, isolate 17; 2, isolate 8 (Bendigo, VIC) from a nasal swab. A and B were derived from the same polyacrylamide 3.5% gel.

12 (Wannamal, WA) and 13 (Katanning, WA) were from farms separated by *c.* 320 km. *CfoI*-generated DNA profiles of these two isolates were similar, although at least six fragments were different (Fig. 4). Isolate 2, cultured from a nasal swab from a pig in Byford, WA, also possessed a non-toxigenic, capsular type A phenotype. Byford is *c.* 95 km from Wannamal. The *CfoI* profiles of DNA from isolates 2 and either 12 or 13 were dissimilar (Fig. 4).

Discussion

Capsular types A and D of *P. multocida* are economically significant pathogens of commercial pig industries because of their ability to induce PAR and their involvement in pneumonia as secondary invaders that

exacerbate lung pathology initiated by primary colonising agents. There have been no reports comparing the genomic profiles generated by REA of isolates from the nasal mucosa and lung lesions of pigs with clinical symptoms of PAR and pneumonia respectively. Although this study compared restriction endonuclease profiles from only a limited number of isolates of *P. multocida*, their origin from lung lesions in sporadic outbreaks of pneumonia and from nasal swabs of pigs in herds displaying symptoms of PAR from different states across Australia sheds light on the degree of genomic heterogeneity inherent in this species. A comparison of the *CfoI* profiles of genomic DNA purified from eight toxigenic type D isolates of *P. multocida* from two geographically distant states (WA and NSW) showed remarkable similarities. The enhanced resolution of the *CfoI* profiles afforded by

Fig. 3. Silver-stained polyacrylamide 3.5% gel showing *CfoI* restriction endonuclease profiles of whole-cell DNA from toxigenic and non-toxicigenic porcine isolates of *P. multocida*. Lane 1, toxigenic, capsular type A isolate 22 (Cootamundra, NSW) from a nasal swab; 2, isolate 18 (Galore, NSW); 3, isolate 19 (Deniliquin, NSW); 4, toxigenic isolate 1 (Byford, WA); 5, isolate 11 from a pneumonic lesion; 6, isolate 5 (Murwillumbah, NSW) from a pneumonic lesion; 7, isolate 14 (Joadja, NSW) from a pneumonic lesion; 8, isolate 6 (Murwillumbah, NSW) from a pneumonic lesion; 9, isolate 9 (Condobolin, NSW) from a pneumonic lesion. Molecular mass markers correspond to lambda DNA digested with *HindIII* (sizes in kb).

polyacrylamide gel electrophoresis and silver staining showed that these eight isolates were clonal. These observations confirm the hypothesis that outbreaks of PAR since 1985 in Australia were initiated from a common infectious source comprising a toxigenic capsular type D strain of *P. multocida* as suggested by Gardner *et al.* [22].

Mercy *et al.* [9] described the first outbreak of PAR in WA after the importation of two sows and it was believed that spread was limited to other states [22]. According to Gardner *et al.* [22], a second outbreak in South Australia (SA) and the associated sale of breeding pigs from this herd to *c.* 131 herds across five Australian states suggested that the SA herd was

the likely source of the toxigenic type D isolate. Gardner *et al.* [22] used REA based on profiles generated by the restriction endonuclease *SmaI*, to describe two different *SmaI* restriction endonuclease profiles (type B or C) amongst 37 Australian toxigenic type D isolates of *P. multocida* (including isolates retyped in this study); all displayed a ribotype 2 profile with the restriction endonuclease *EcoRI*. However, the only difference between *SmaI* profile types B and C resides in the absence of a single DNA fragment *c.* 1.9 kb which was likely to have been derived from plasmid DNA because of the relatively high staining intensity compared with genomic DNA fragments. Similarly, the present study noted the absence of a darkly staining fragment of *c.* 4.4 kb in the *CfoI* profile of isolate 21 which is likely to have arisen from a restriction fragment length polymorphism (RFLP) in plasmid DNA. Plasmids are known to exist in all seven toxigenic, capsular type D strains in this study (data not shown and [22]). This observation is supported by the inability to detect any other RFLPs in the *CfoI* profile throughout the entire molecular mass range resolvable with a polyacrylamide 3.5% matrix. The presence of a RFLP in the plasmid profiles amongst these isolates is likely to be responsible for the *SmaI* profiles (types B and C) described previously [22]. The results in Figs. 2 and 3 show that the *CfoI* restriction profiles of the eight capsular type D toxigenic isolates are derived from a single clonal type which probably emanated from the original outbreak of PAR in WA. These data suggest that the second outbreak in SA was probably caused by a type D toxigenic isolate which first appeared in WA.

A single toxigenic capsular type A isolate of *P. multocida* (isolate 22) displayed a unique *CfoI* profile. This observation is supported by the findings of Gardner *et al.* [22] who demonstrated that this isolate produced a unique *SmaI* profile (type G, ribotype 3) unlike the toxigenic type D isolates.

Two non-toxicigenic *P. multocida* isolates from nasal swabs of pigs in a herd displaying clinical symptoms of PAR (2 and 3) showed distinct and highly variable *CfoI* profiles, suggesting a limited role in the aetiology of PAR. However, non-toxicigenic strains of *P. multocida* may be ubiquitous in the nasal cavities of pigs [28, 29] and their ability to colonise the lower respiratory tract and their contribution to the development of pasteurella pneumonia is unknown. Previous studies with European isolates of *P. multocida* have reported that the vast majority of non-toxicigenic isolates do not possess the *toxA* gene, although a few isolates do possess highly mutated or incomplete *toxA* sequences [4]. The *toxA* gene or parts thereof could not be detected in Southern blots (probed with *pPmF3.5*) of whole-cell DNA purified from the 10 pneumonic isolates nor two nasal swab isolates (isolates 2 and 3) used in this study.

Fig. 4. Silver-stained polyacrylamide 3.5% gel showing *Cfo*I restriction endonuclease profiles of whole-cell DNA purified from non-toxicogenic isolates of *P. multocida*. Lane 1, isolate 3 (Byford, WA) from a nasal swab; 2, isolate 4 (Murwillumbah) from a pneumonic lesion; 3, isolate 6 (Murwillumbah) from a pneumonic lesion; 4 and 5, isolate 7 from a pneumonic lesion; 6, isolate 10 from a pneumonic lesion; 7, isolate 12 (Wannamal, WA) from a pneumonic lesion; 8, isolate 13 (Katanning, WA) from a pneumonic lesion; 9, isolate 2 (Byford, WA) from a nasal swab; 10, isolate 12; 11, molecular mass markers (lambda DNA digested with *Hind*III).

Although *P. multocida* has been recognised as an important pathogen in the development of PAR, its role in the aetiology of swine pneumonia is unclear. It is generally considered that *P. multocida* is not a primary colonising agent of the swine respiratory tract and initial infection with *Mycoplasma hyopneumoniae* [30, 31] or one of several viruses including adenovirus [32], hog cholera virus [33] or pseudorabies virus [34] is required for subsequent infection with *P. multocida*. In Australia, with the exception of *M. hyopneumoniae*, none of these agents is present. Pijoan and Fuentes [13] described the isolation of non-toxicogenic capsular type A isolates from swine with symptoms of severe pleuritis and abscess formation. In that study, several non-toxicogenic, capsular type A isolates, as well as one toxicogenic, capsular type D isolate, were used in conjunction with pseudorabies virus to induce experimental pneumonia and pleuritis in pigs. Certain non-toxicogenic, capsular type A isolates were more capable of inducing severe pleuritis and abscess formation than other isolates and the toxicogenic, capsular type D isolate seemed unable to exacerbate the pathological

changes. Studies by Iwamatsu and Sawada [14] described 116 *P. multocida* isolates from lung lesions in Nakasaki, Japan. Capsular type D, toxicogenic isolates (18.1%) were isolated only from abscesses, whilst the majority of isolates were capsular type A (81.9%) and were from lungs of pigs with pleuritis and consolidation. Although these studies suggest that capsular type A isolates of *P. multocida* are associated with pneumonic lesions in pigs, there have been no reports comparing the genetic relatedness of such isolates from sporadic outbreaks of pneumonia.

In the present study, DNA was purified from whole-cell lysates of 10 isolates (five each of capsular types A and D) of *P. multocida* from lung lesions. These were from sporadic outbreaks of pneumonia mostly in piggeries located in different areas of NSW and WA. Unlike the *Cfo*I profiles of the toxicogenic capsular type D isolates, these 10 isolates generally displayed heterogeneous *Cfo*I profiles both amongst themselves and when compared with 12 isolates from the nasal passages of pigs. None of the 10 lung isolates

expressed cytotoxin and all failed to show the presence of plasmid DNA (data not shown). These data suggest that opportunist *P. multocida* are associated with pneumonic pasteurellosis in swine. The inability of pneumonic isolates to reproduce clinical symptoms of respiratory disease in experimental challenge models in pigs without concomitant infection with *M. hyopneumoniae* or a viral agent [30–34] and the ability to recover simultaneously several different genotypes from the same animal are consistent with this hypothesis. Nevertheless, it is interesting that all pneumonic isolates of *P. multocida* in the present study did not possess the *tox*A gene. It is likely that toxin production does not play a role in the pathogenesis of pneumonia or pleuritis. Multilocus enzyme electrophoretic studies with 35 disease isolates of *P. trehalosi*, a pathogen causing systemic infection in the upper alimentary tract and lungs of sheep, identified 16 different electrophoretic types indicative of an opportunist mechanism of pathogenicity [35]. Further studies are required to determine the degree of genetic heterogeneity amongst a larger number of isolates of *P. multocida* associated with pneumonia. Finally, polyacrylamide gel electrophoresis and silver staining produce highly informative DNA fingerprints which should be useful in studies tracing outbreaks of PAR and pneumonia in swine.

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