Characterisation of porcine haemophili isolated from Australian pigs between 1988 and 1992

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SUMMARY: A total of 362 haemophili, isolated from pigs throughout Australia, were characterised by phenotypic properties. Most were identified as *Actinobacillus pleuropneumoniae* (296 isolates) or *Haemophilus parasuis* (52 isolates). The remaining isolates were identified as *Haemophilus* Taxon 'minor group' (12 isolates) and *Haemophilus* Taxon D (two isolates). All 296 *A pleuropneumoniae* isolates were serotyped by slide agglutination and/or gel diffusion, using rabbit antisera against all 12 recognised serovars. Of these, only 156 (52.7%) could be assigned to a single serovar as follows: serovar 1 - 85 isolates, serovar 2 - 4 isolates, serovar 3 - 2 isolates, serovar 5 - 10 isolates, serovar 7 - 51 isolates, serovar 11 - 2 isolates and serovar 12 - 2 isolates. Of the remaining 140 isolates, 91 gave cross-reactions with serovars 3 and 6, one cross-reacted with serovars 9 and 10, one cross-reacted with serovars 9 and 11 whereas 47 gave no reaction with any of the antisera.

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Introduction

Haemophili can be defined as bacteria that have an in-vitro requirement for one or both of two growth factors - haemin (X-factor) and nicotinamide adenine dinucleotide (NAD; V-factor) (Eaves *et al* 1989). Most of the known haemophili from pigs require the V-factor for growth *in vitro* (Nicolet 1986). The exception is the species *Actinobacillus pleuropneumoniae*, which contains both V-factor-dependent strains, termed biovar 1, and V-factor-independent strains, termed biovar 2 (Pohl *et al* 1983). Other porcine haemophili include *Haemophilus parasuis* and a range of other taxa not yet assigned to a species; *Haemophilus* Taxa C, D, E and F as well as Taxa 'minor group' and 'urease negative' (Eaves *et al* 1989; Møller and Kilian 1990).

Two economically important diseases associated with the porcine haemophili are porcine pleuropneumonia, caused by *A pleuropneumoniae*, and Glasser's disease, caused by *H parasuis* (Nicolet 1986). Of these two diseases, porcine pleuropneumonia, a severe respiratory disease characterised by necrotising fibrinohaemorrhagic pneumonia with fibrinous pleurisy (Nicolet 1986), is of greater economic significance in Australia. The disease occurs world-wide and is recognised as a cause of substantial economic losses (Mittal *et al* 1992).

Currently 12 serovars of *A pleuropneumoniae* are recognised with the prevalence of these serovars varying from country to country (Mittal *et al* 1992). A knowledge of serovar distribution is important in the application of vaccines, because inactivated vaccines protect only against those serovars within them (Nielsen 1976, 1984).

A national referral service, provided by this Institute, for the identification of porcine haemophili, is supported by the Australian Pig Research and Development Corporation. We have published earlier results generated by this service (Eaves and Blackall 1988; Eaves *et al* 1989). In this report, we describe the phenotypic characterisation of 362 haemophili isolated between mid-1988 and mid-1992 and the serological characterisation of the 296 isolates identified as *A pleuropneumoniae*. We also establish the widespread occurrence of *Haemophilus* Taxon 'minor group' in Australian pigs. In the serological study we report on the recognition of two new serovars of *A pleuropneumoniae* in Australian pigs and the widespread occurrence of *A pleuropneumoniae* isolates that cannot be confidently assigned to a single serovar.

Materials and Methods

Isolates

The 362 haemophili were obtained from diagnostic veterinary laboratories located in New South Wales (68 isolates), Queensland (93), South Australia (45), Tasmania (2), Victoria (124) and Western Australia (30). The reference strains used were as follows: *A pleuropneumoniae* serovars 1 to 12 (strains 4074, 4226, 1421, M62, K17, Femo, WF83, 405, CVJ 13621, 22009, 56153 and 1096, respectively), the type strain for *A pleuropneumoniae* biovar 2 (strain 20086/76), *H parasuis* Bakos serovars A to D (strains A9, B26, C5, D74, respectively), *H parasuis* Nicolet serovars 1 to 5 (strains NR40, SW140, SW114, SW124 and Nagasaki, respectively), *Haemophilus* Taxa 'minor group' (strains CP109III, CP 215 VI and 202), C (strains CAPM 5111 and CAPM 5113), D (strains ME-14, SP-62), E (strains B-20 and $27KC_{10}$) and F (strains NM-314 and 37E3).

Media

TM/SN, a medium capable of supporting the growth of V-factor-dependent haemophili, was prepared as described previously (Reid and Blackall 1987), and used to grow inocula for biochemical tests and antigens for serological testing. Other media are described in the following sections. Incubation, unless otherwise indicated, was at 37°C in air containing 5% carbon dioxide.

Characterisation Tests

Tests for Gram stain reaction, catalase, urease, ONPG and requirements for the growth factors, X and V, were performed as described previously (Blackall and Reid 1982). The ability to produce acid from arabinose, glucose, lactose, mannitol, sucrose and xylose was tested on all isolates using a replica plating technique (Blackall 1983). For those isolates that were urease negative, the ability to produce acid from galactose, inositol, maltose, mannose, melibiose, raffinose and sorbitol was also determined by the replica plating method. The indole reaction of the urease negative strains was determined using tryptone water supplemented with reduced NAD (0.0025% w/v), chicken serum (1% v/v), thiamine (0.0005% w/v) and oleic-albumin complex (5% v/v).

TABLE 1
Geographical distribution of isolates of porcine haemophili

State	*A pleuropneumoniae*	*H parasuis*	Taxon 'minor group'	Taxon D
New South Wales	53	13	1	1
Queensland	78	13	2	0
South Australia	35	5	5	0
Tasmania	0	2	0	0
Victoria	107	16	0	1
Western Australia	23	3	4	0
TOTAL	296	52	12	2

TABLE 2
Geographical origin and serovar of 296 Australian isolates of *Actinobacillus pleuropneumoniae*[*]

Origin	Serovar										
	1	2	3	5	7	11	12	NR[†]	3,6	9,10	9,11
New South Wales	2	–	–	4	18	–	1	6	22	–	–
Queensland	4	3	1	3	11	1	1	15	38	–	1
South Australia	7	–	1	–	6	–	–	10	11	–	–
Victoria	63	–	–	3	10	1	–	13	16	1	–
Western Australia	9	1	–	–	6	–	–	3	4	–	–
TOTAL	85	4	2	10	51	2	2	47	91	1	1

* The table presents the combined results of both the rapid slide agglutination or gel diffusion tests

† No reaction with any antiserum in either the rapid slide agglutination or gel diffusion tests

TABLE 3
Distribution of source of *Actinobacillus pleuropneumoniae* serovars between on farm disease investigations and abattoir checks

Serovar	Number	Number of isolates obtained from		
		Disease investigation	Abattoir check	Unknown
1	85	49	6	30
2	4	1	1	2
3	2	0	0	2
5	10	2	2	6
7	51	11	4	36
11	2	0	1	1
12	2	0	1	1
3, 6	9	40	26	25
9, 10	1	1	0	0
9, 11	1	1	0	0
NR[*]	47	14	13	20
Total	296	119	54	123

* No reaction with any antiserum.

Antisera

The antisera for serovars 1 to 8 were those used in a previous study (Eaves and Blackall 1988). The antisera for serovars 9 to 12 were produced as described previously (Eaves and Blackall 1988).

Serotyping Tests

Two serotyping tests were used. All the isolates were examined using a modification of the rapid slide agglutination (RSA) test described by Rapp *et al* (1985). Briefly, the isolates were grown on TM/SN agar at 37°C for 5 h. The growth was then harvested into 2 mL of 0.15M NaCl. Equal volumes of this antigen suspension and undiluted antisera were then mixed. Agglutination was recorded within 5 min. If only a single antiserum reacted this was accepted as the serovar of the isolate.

Isolates that were either nontypable, or autoagglutinating, or reacted only with antiserum to serovar 3 or gave cross-reactions in the RSA test were also tested using a modification of the gel diffusion (GD) test described by Gunnarsson (1979). Briefly, the antigen suspension prepared for the RSA test was centrifuged and the wet weight of the cell pellet determined. The cells were resuspended in sterile distilled water (2 mL for each 0.1 g of wet cells) and an equal volume of phenol added. The mixture was then heated at 65°C for 20 min under magnetic stirring, centrifuged (14 000 × *g*, 5 min) and the supernatant dialysed against distilled water at 4°C overnight. The antigen was then used in the GD test using agarose in Veronal buffer. The GD result was accepted as the definitive result for all isolates tested by this method.

Results

All 362 Australian isolates were Gram negative rods with a requirement for V-, but not, X-factor. Of these, 296 isolates were identified as *A pleuropneumoniae* biovar 1, 52 were identified as *H parasuis*, 12 as *Haemophilus* Taxon 'minor group' and 2 as *Haemophilus* Taxon D. The geographical distribution of the isolates is given in Table 1.

The serotypes of the 296 isolates of *A pleuropneumoniae* are shown in Table 2, which presents the combined results for both the RSA and GD tests. Serovars 1, 5 and 7 were the only serovars that were confidently identified by the RSA test. All the other results presented in Table 2 are based on the GD test. Only 156 of the 296 isolates could be assigned to a serovar. The commonest serovar was serovar 1 (85 isolates) followed by serovars 7 (51 isolates), 5 (10 isolates), 2 (4 isolates), 3 (two isolates), 11 (two isolates) and 12 (two isolates). A total of 140 of the isolates could not be confidently serotyped with 91 reacting with serovars 3 and 6, one reacting with serovars 9 and 10, one reacting with serovars 9 and 11, and 47 not reacting at all.

Isolates of serovars 1 and 7 were derived from five States. Serovar 5 was also widely distributed, occurring in Queensland, New South Wales and Victoria. The remaining serovars, 2, 3, 11 and 12, were found in small numbers only and had limited distribution.

The isolates that reacted with serovars 3 and 6 or failed to react at all were derived from five States and outnumbered the isolates that could be serotyped in Queensland, New South Wales and South Australia.

For 173 of the 296 *A pleuropneumoniae* isolates, the information submitted with the isolates was sufficient to classify them according to whether they were obtained from an outbreak of clinical disease on a farm or from a lung examination at slaughter (Table 3).

Discussion

This study confirmed the presence of four species among Australian isolates of porcine haemophili; *A pleuropneumoniae* biovar 1, *H parasuis*, *Haemophilus* Taxon 'minor group' and *Haemophilus* Taxon D. In our initial characterisation of 70 earlier isolates, we detected only *A pleuropneumoniae* and *H parasuis* (Eaves *et al* 1989). Subsequently, we reported the isolation and characterisation

of two of the 12 *Haemophilus* Taxon 'minor group' isolates (Blackall *et al* 1991) and the two *Haemophilus* Taxon D isolates (Blackall *et al* 1994) reported here. These previously described cases are included in Table 1.

Our current study has demonstrated that *Haemophilus* Taxon 'minor group' is widely distributed in Australia, isolates having being obtained from pigs in New South Wales, Queensland, South Australia and Western Australia. As we reported previously (Stephens *et al* 1990), the role of *Haemophilus* Taxon 'minor group' in porcine respiratory disease remains unclear. The overall evidence is that the organism is unlikely to cause disease. Similarly, we have noted that the role of Taxon D in disease is uncertain (Blackall *et al* 1994).

Our finding that isolates of *Haemophilus* Taxon 'minor group' and Taxon D can be obtained from Australian pigs has important implications for diagnostic laboratories serving the industry. Simplified identification schemes that do not involve carbohydrate fermentation patterns do not allow the separation of *A pleuropneumoniae* and *Haemophilus* Taxon 'minor group' or *H parasuis* from *Haemophilus* Taxa C, D, E or F. The recognition that at least four different taxa of haemophili exist in Australian pigs increases the need for complete biochemical characterisation of all isolates of these organisms.

A range of different tests has been used to serotype isolates of *A pleuropneumoniae* including tube agglutination, GD, RSA and indirect haemagglutination tests and they have been reviewed recently (Mittal *et al* 1992). We chose the RSA and GD tests as we previously found they combine ease of use and specificity (Eaves and Blackall 1988). The two tests did not perform well in the current study.

Previous serotyping studies have demonstrated that serovars 1, 2, 3, 5 and 7 of *A pleuropneumoniae* are present (Eaves and Blackall 1988; Stephens *et al* 1990). The current study extends this knowledge and establishes the presence of two new serovars, 11 and 12. Serovar 1 was again the commonest serovar with serovar 7 next. We have also established that *A pleuropneumoniae* serovar 5 is present in Queensland, New South Wales and Victorian pigs. This serovar was first isolated from pigs in Queensland in 1990 (Stephens *et al* 1990), and though there were only 10 isolates, they represented the third most frequently identified serovar after serovars 1 and 7.

A pleuropneumoniae isolates from pigs in Victoria have not been serotyped previously. The results for the Victorian isolates reflects the national trend with serovars 1, 7 and 5 being the most common serovars, in decreasing order. The dominance of serovar 1 in Victoria is not reflected in Queensland and New South Wales where serovar 7 was the most common serovar.

A notable finding of the current study was the large percentage of isolates that could not be assigned to a serovar. Almost half the isolates were either nontypable (47) or reacted with antisera to serovars 3 and 6 (91). The problem of cross-reactions involving serovars 3, 6 and 8 has been noted by others (Rapp *et al* 1985; Mittal *et al* 1988). While these serovars do possess serovar-specific antigens they also possess cross-reacting antigens and no single serotyping method appears to be suitable for these serovars. Mittal *et al* (1988) recommended that coagglutination and a quantitative immunodiffusion test should be used.

We believe that there are two possible explanations for the serovar 3 and 6 cross-reacting isolates revealed in this study. The first explanation is that the isolates truly belong to either serovars 3 or 6 and the serotyping methods we used cannot assign the isolates to the correct serovar. Further work with additional tests, such as the coagglutination test, the quantitative immunodiffusion test and the indirect haemagglutination test, is needed to test this. The second possible explanation is that the cross-reacting isolates are members of another serovar, or serovars, which have a degree of antigenic similarity with serovars 3 and 6. There is some evidence to support this possibility. A recent study on the genetic diversity of Australian isolates of *A pleuropneumoniae* has included some of these cross-reacting isolates and indicated that the cross-reacting isolates examined represented several different clonal lines that were not closely related to serovars 3, 6 or 8 (Hampson *et al* 1993).

It is important that the non-reacting and cross-reacting isolates described in this study are further characterised. There is evidence that inactivated vaccines protect only against challenge from the homologous serovar (Nielsen 1976, 1984). Hence, resolution of the status of the cross-reacting and non-typable isolates is a priority if effective vaccination programmes are to be developed in Australia.

The data on the association of the various serovars with either disease investigations or abattoir checks are limited by a lack of information supplied with many isolates. However, some tentative conclusions can be drawn. Serovar 1 is strongly associated with disease outbreaks on farms (49 disease-associated isolates compared with six isolates from abattoir checks). In contrast, no strong association is apparent with the non-typable isolates or those that cross-react with serovars 3 and 6. Of the 66 isolates that cross-reacted with serovars 3 and 6 and were submitted with detailed information, almost one-third came from abattoir checks. Similarly, almost half of the 27 non-typable isolates of known origin were obtained at abattoir checks. These results suggest that, at least for some of the serovar 3 and 6 cross-reacting isolates and some of the non-typable isolates, there might have been no strong clinical evidence of infection in the herd. There is evidence that serovars of *A pleuropneumoniae* differ in pathogenicity. Previous work at this Institute demonstrated that in Australia, serovar 1 isolates are far more pathogenic than isolates of serovars 2, 3 and 7 (Rogers *et al* 1990). Isolates of all four serovars caused typical lung lesions of porcine pleuropneumonia, but isolates of serovar 1 killed 12 out of 16 pigs challenged, whereas isolates of the other serovars killed only one of the 40 pigs challenged (Rogers *et al* 1990). There may be considerable difference in the pathogenic potential of the *A pleuropneumoniae* isolates currently grouped together as "cross-reacting with serovars 3 and 6". Some of these isolates may cause frank disease and deaths in animals while others are detected only by the presence of lesions at slaughter. Recognition that only certain isolates are associated with clinical outbreaks of pleuropneumonia must await resolution of the status of the serovar 3 and 6 cross-reacting isolates.

Compared with the previous serotyping study (Eaves and Blackall 1988), we recorded a large proportion of isolates that are either non-typable or cross-reacting with serovars 3 and 6. A possible explanation for this increase relates to the fact that, during the current study, pig health monitoring schemes based on the examination of pig viscera at abattoirs were widely adopted by the Australian pig industry. Hence, many diagnostic laboratories in Australia received submissions from pneumonic lungs detected at slaughter. As shown in Table 3, the serovar 3 and 6 cross-reacting isolates and non-typable isolates were more frequently represented in isolates from slaughter samplings than isolates of serovar 1. It is possible that the specific monitoring of pigs at slaughter for pneumonia and pleurisy resulted in greater efforts to isolate the cause of pneumonic lesions.

In summary, this study has confirmed that serovar 1 continues to be the predominant serovar of *A pleuropneumoniae* isolated from Australian pigs. The next most common serovars identified were serovars 7 and 5. Almost half of the isolates examined could not be assigned to a serovar, either cross-reacting with serovars 3 and 6 or giving no reaction at all. The relevance, identification and pathogenicity of these non-reacting and cross-reacting isolates requires resolution.

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