# Characterisation of haemolytic RTX toxins produced by Australian isolates of *Actinobacillus pleuropneumoniae*

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**SUMMARY: The haemolytic RTX toxins of 27 isolates of *Actinobacillus pleuropneumoniae*, representing all serovars that have been isolated in Australia, were characterised. The quantity of protein secreted by these isolates into the media was not significantly different between serovars, but haemolytic activity was detected only in the unconcentrated supernatants from cultures of serovar 1 and 5 isolates. Haemolytic activity in supernatants of serovar 2, 3 and 7 isolates was detected only after the supernatants were concentrated. On Southern hybridisation blots, genomic DNA of serovar 1 and 5 isolates contained regions that were similar to the cloned structural genes for ApxI (*apxIA*) and for ApxII (*apxIIA*). In contrast, genomic DNA of serovar 2, 3 and 7 isolates only contained regions similar to, if not identical with, the cloned *apxIIA* gene. The haemolytic activity of the culture supernatant depends on the type or composition of media and adaptability of the bacteria to in-vitro cultivation. Low passage cultures of *A pleuropneumoniae*, which were characterised by waxy colonies, produced significantly weaker haemolytic activity than *A pleuropneumoniae* after several passages *in vitro*.**

*Aust Vet J* **73**: 164 – 169

## Introduction

*Actinobacillus pleuropneumoniae* is the causative agent of contagious pleuropneumonia, which causes significant economic losses to the pig industry world-wide. The first reported outbreak of this disease in Australia occurred in New South Wales in early 1970s (Mylrea *et al* 1974). Subsequent outbreaks were reported in Queensland (Cameron and Kelly 1979) and in Tasmania (Mason *et al* 1982). So far, five serovars, No. 1, 2, 3, 5 and 7, are known to exist in this country, of which, serovars 1 and 7 are the most prevalent (Eaves and Blackall 1988; Stephens *et al* 1990).

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*A pleuropneumoniae* produces cytotoxins, which are thought to be important, if not the most important, factors for bacterial virulence and pathogenicity of the disease. Inoculation of pigs with bacteria-free culture supernatants produces lesions in the lungs similar to those produced by live *A pleuropneumoniae* (Rosendal *et al* 1980). A non-haemolytic mutant of *A pleuropneumoniae* derived from a virulent strain of serovar 5 failed to cause disease when inoculated into mice or pigs (Inzana *et al* 1991). In addition, immunisation of mice or pigs with the mutant strain did not protect the animals against lethal infection.

According to a new standardised nomenclature, cytotoxins (RTX toxins) of *A pleuropneumoniae* are called Apx (Frey *et al* 1993). Three different Apx secreted by the 12 serovars of *A pleuropneumoniae* have been identified. ApxI, formerly called haemolysin-I or cytolysin-I, is strongly haemolytic and strongly cytotoxic; ApxII,

formerly called haemolysin-II or cytolysin-II, is weakly haemolytic and moderately cytotoxic; and ApxIII, formerly called cytolysin-III or pleurotoxin, is not haemolytic, but is strongly cytotoxic (Kamp *et al* 1991).

The pathogenicity and antimicrobial sensitivity of the Australian isolates of *A pleuropneumoniae* have been described (Eaves *et al* 1989; Rogers *et al* 1990). In this study we characterised haemolytic Apx produced by the Australian isolates of *A pleuropneumoniae*.

## Materials and Methods

### *Bacterial Isolates*

Twenty-seven isolates of *A pleuropneumoniae* used in this study were originally isolated from pigs in various parts of Australia. These isolates were serotyped in a previous study using rapid slide agglutination and gel diffusion tests with serum from rabbits immunised with appropriate *A pleuropneumoniae* reference strains (Eaves and Blackall 1988).

### *Preparation of Apx*

*A pleuropneumoniae* isolates were grown on chocolate blood agar (CBA) plates at 37°C for about 18 h in a candle jar. Bacteria from each CBA plate were suspended in 15 mL of RPMI-1640[‡] with or without supplementation. After incubation at 37°C for 2 h, the bacteria were pelleted by centrifugation at 12 000 *g* for 10 min, and the supernatant was collected and put on ice until its haemolytic activity was assayed. In some experiments, the culture medium (RPMI-1640) was supplemented with either $CaCl_2$ (10 mM), ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid[§] (EGTA, 2.2 mM) to chelate $Ca^{2+}$, swine serum albumin[§] (10 mg/mL), normal pig serum (10%) or pig erythrocytes ($10^6$/mL) lysed with distilled water.

Alternatively, Apx were produced in broth media as previously described (Frey and Nicolet 1988). Briefly, Colombia broth[¶] supplemented with 1% IsoVitaleX[¶] and 10 µg/mL of β-NAD[§]; or brain heart infusion broth[#] supplemented with 10 µg/mL of β-NAD was inoculated with *A pleuropneumoniae* and grown at 37°C. At mid-exponential growth phase, determined turbidometrically ($A_{620nm}$ = 0.8), the bacteria were pelleted by centrifugation and the supernatant was collected.

Concentration of the toxins to about 1/300 of the original supernatant volume was achieved by an ammonium sulphate precipitation technique. The toxins were precipitated by slowly adding ammonium sulphate to 80% saturation, followed by centrifugation at 15 000 *g* for 30 min. Before addition of the ammonium sulphate, bovine serum albumin[§] was added to the supernatant to a final concentration of 0.5 mg/mL in order to stabilise the toxins and facilitate their concentration. The precipitate was dissolved in RPMI-1640 and undissolved materials were removed by centrifugation at 15 000 *g* for 30 min. Desalting and further concentration were achieved by ultrafiltration using Centriprep-10 concentrators[**]. The concentrated Apx were stored at -70°C.

### *Assays for Haemolytic Activity and Protein Concentration*

Serial two-fold dilutions of haemolysin in 100 µL RPMI-1640, or other media when indicated, were prepared in 96-well, round-bottomed microplates. In addition, RPMI-1640 and 0.5% Triton X-100[§] were used as controls for 0% and 100% lysis, respectively. After addition of 100 µL sheep red blood cell (RBC) suspension (1% in phosphate buffered saline [PBS], pH 7.4) to each well, the plate was incubated at 37°C for 2 h. After incubation, unlysed RBC were pelleted by centrifugation at 700 *g* for 5 min. Some of the supernatant (125 µL) was transferred to a 96-well, flat-bottomed microplate, and the optical density ($A_{540\ nm}$) of the supernatant was measured in a microplate reader. The degree of haemolysis (% haemolysis) was calculated as follows:

$$[(A_{540nm}\text{sample} - A_{540nm}\text{RPMI-1640})/(A_{540nm}\text{Triton X-100}) - (A_{540nm}\ \text{RPMI-1640})] \times 100$$

$A_{540nm}$ RPMI-1640 and $A_{540nm}$ Triton X-100 represent the absorbance of 0% and 100% lysis, respectively. One haemolytic unit (HU) was defined as the lowest concentration of the Apx preparation causing 50% haemolysis, determined by plotting the sample % haemolysis against dilution on semilog graph paper.

Protein concentration in the supernatant of cultures before and after incubation was determined according to the Bradford method using a commercial kit[††]. Bovine IgG[††] was used as an assay standard. The difference between the protein concentration of supernatants of bacterial cultures before and after incubation was referred to as the amount of secreted protein.

### *Identification of Structural Apx Genes*

Genes encoding the structural protein of haemolytic Apx in the *A pleuropneumoniae* field isolates were compared with the gene encoding structural proteins of ApxI (*apx*IA) and ApxII (*apx*IIA) of the reference strain (S4707) of serovar 1. Plasmids pJFF 729 and pJFF727 containing *apx*IA and *apx*IIA genes, respectively (Frey *et al* 1992) were amplified in *Escherichia coli* (strain DH5α), labelled with [α-$^{32}$P]-dCTP[‡‡] using a random primed DNA labelling kit[§§] and used as probes in Southern blot analysis (Sambrook *et al* 1989).

Bacterial genomic DNA, purified as described by Johnson (1991), was digested with the restriction endonucleases *Pst*I, *Pst*I and *Hin*dIII, *Pst*I and *Sac*I, *Kpn*I and *Eco*RV, *Kpn*I and *Hind*III or *Eco*RV and *Hind*III[§§]. The DNA fragments were separated by electrophoresis in 0.7% agarose before transfer to nylon membranes (Hybond-N[‡‡]). Prehybridisation (2 h) and hybridisation (overnight) were performed at 55°C in 6 x SSC (6 x 0.15 M NaCl plus 6 x 0.015 M sodium citrate), 0.5% sodium dodecyl sulfate (SDS), 5% Denhardt's solution and 50 µg/mL salmon sperm DNA. The blots were washed twice in conditions of medium stringency (0.1 x SSC, 0.5% SDS at 55°C for 30 min each washing) and then autoradiographed.

### *Statistical Analysis*

The amounts of protein secreted by isolates carrying *apxIA* gene (serovar 1 and 5 isolates) were compared with those secreted by other isolates (serovar 2, 3 and 7 isolates) using a *t*-test. The production of haemolytic Apx by organisms from waxy-type colonies was compared with that by organism from soft-type colonies of the same isolates using a *t*-test. Effects of suplementation of culture media with either albumin, serum or erythrocyte lysate were analysed using a one-way analysis of variance. Data for the colony-type and culture-supplementation experiments were derived from 3 separate assays (3 different supernatants). A probability value (P) 0.05 was considered to be significant.

## Results

Haemolytic activity was detected only in the unconcentrated supernatants of serovar 1 and 5 isolates (Figure 1). The activity was never detected in the similar culture supernatants from serovar 2, 3 and 7 isolates. Even after the supernatants of these isolates were concen-

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¶ BBL, Microbiology System, Cockeysville, MD, USA
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** Amicon Co., Denvers, MA, USA
†† Bio-Rad Laboratories, Richmond, CA, USA
‡‡ Amersham Australia Pty Ltd, Sydney, Australia
§§ Boehringer Mannheim Australia Pty Ltd, Castle Hill, NSW, Australia

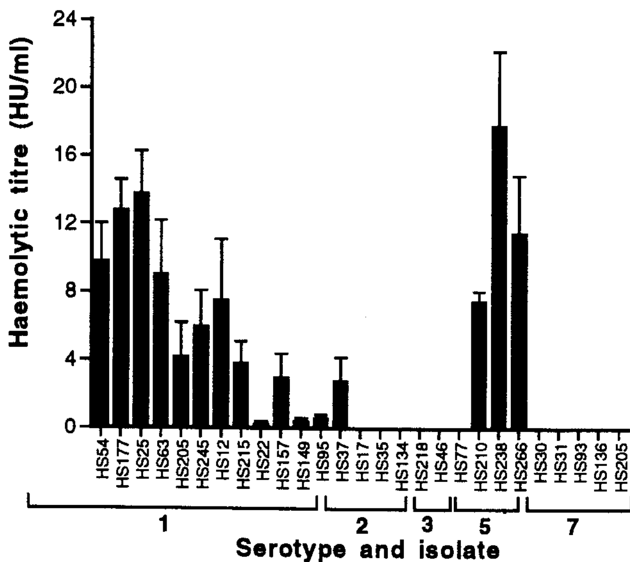



Figure 1. Haemolytic activity of bacteria-free culture supernatants of Australian isolates of *A pleuropneumoniae* grown overnight on chocolate blood agar and suspended in RPMI-1640. (mean ± SE of 3 supernatants).

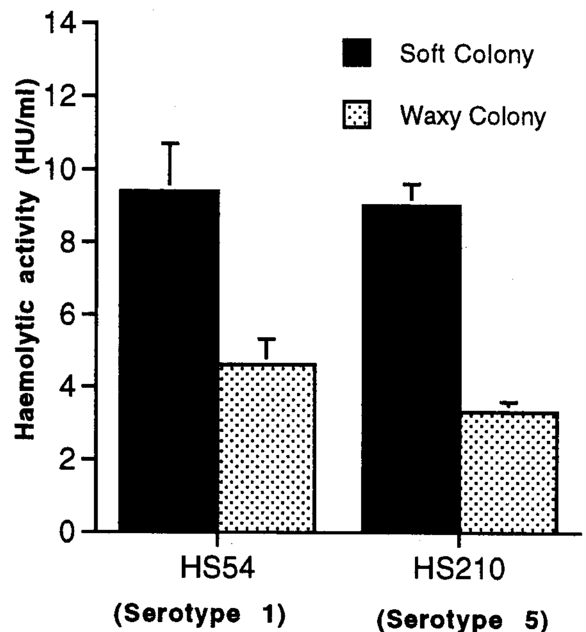



Figure 2. Haemolytic activity of bacteria-free culture supernatants from *A pleuropneumoniae* derived from soft, glistening colonies and organisms from dense, waxy colonies. (mean ± SE values of 3 supernatants). * $P < 0.01$.

trated 300-fold, only weak haemolysis was observed. The weaker haemolytic activity in the supernatants from serovar 2, 3, and 7 isolates was not because less protein was produced in culture. The amount of protein secreted by the serovar 1 and 5 isolates was not different ($P > 0.05$) from that secreted by the serovar 2, 3 and 7 isolates (data not shown).

There was high variability in the haemolytic activity between isolates within a serovar and also between colonies within an isolate. *A pleuropneumoniae* often had two distinct types of colonies on CBA. One colony was waxy, strongly adherent, and difficult to remove from the CBA, whereas the other, was soft and easily removed from the CBA. Organisms from soft colonies had consistently greater haemolytic activity than those from the waxy colonies (Figure 2).

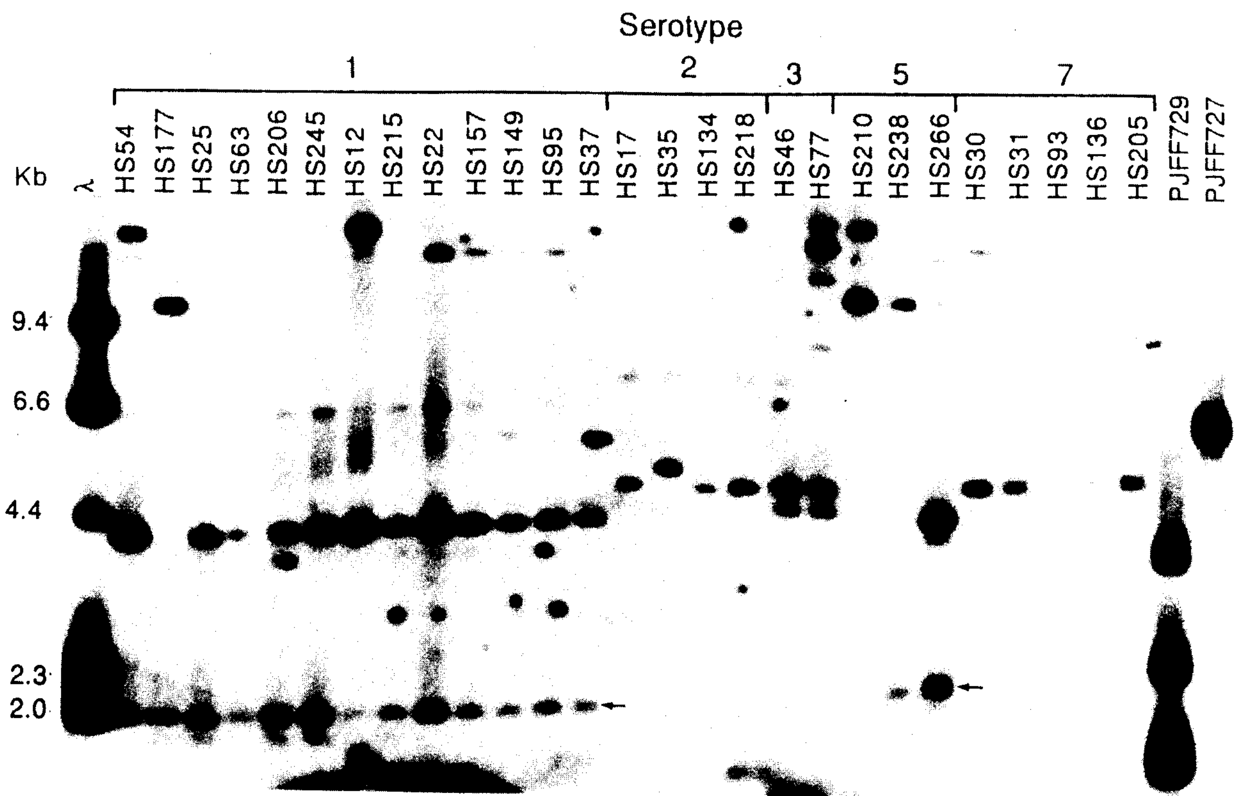
The production of haemolytic Apx was also dependent on the type of media used. Haemolysins were produced more consistently when *A pleuropneumoniae* was cultured on CBA, followed by suspension and incubation of the bacteria in RPMI-1640. Suspension of the bacteria in broth media (Colombia broth or brain heart infusion broth) resulted in inconsistent production of haemolytic Apx, and haemolytic activity in the supernatant was often very low or undetectable. The reason for the low or undetectable haemolytic activity was not because of the presence of any substance in the broth that inactivated the haemolytic Apx. Three representative isolates of *A pleuropneumoniae* grown overnight on CBA, followed by suspension and brief (2 h) incubation in Colombia broth, produced Apx with haemolytic activity comparable to that if suspended and incubated in RPMI-1640 (data not shown). *A pleuropneumoniae* grown overnight on CBA followed by suspension and incubation in PBS (pH 7.4) or Tris buffered saline (10 mM Tris, 150 mM NaCl, pH 7.4) supplemented with 10 mM $CaCl_2$, did not produce detectable amounts of haemolytic Apx. This indicates that the toxins were not synthesised and secreted during growth on CBA.

With the same isolates, supplementation of RPMI-1640 with erythrocyte lysate significantly increased ($P < 0.05$) the haemolytic activity of culture supernatant of all 3 isolates examined, and supplementation with swine serum increased the haemolytic activity of 2 isolates, but supplementation with swine albumin had no effect in any of the 3 isolates examined. Supplementation of Columbia broth with swine serum or albumin did not significantly increase the haemolytic activity of culture supernatants from the 3 isolates, and supplementation of this broth with erythrocyte lysate increased the haemolytic activity in culture supernatant from only 1 isolate (data not shown).

Haemolytic activity was undetectable in supernatants when Colombia broth was not supplemented with $Ca^{2+}$ ( $CaCl_2$) or when $Ca^{2+}$ in the RPMI-1640 was chelated with 2.2 mM EGTA. However, addition of $Ca^{2+}$ ($CaCl_2$) to RPMI-1640 ( to a final concentration of 5 mM or 10 mM) did not increase haemolytic activity in the supernatant of any isolates. Unlike Apx of serovar 1 and 5 isolates, Apx of serovar 2, 3 and 7 isolates required $Ca^{2+}$ in the assay media as a cofactor to lyse erythrocytes. Concentrated Apx preparations derived from serovar 2, 3 and 7 isolates were unable to lyse erythrocytes when $Ca^{2+}$ from assay media was chelated with EGTA, or when PBS or Tris buffered saline that was not supplemented with $Ca^{2+}$ was used as assay media (data not shown).

Southern hybridisation analyses indicated that the genomic DNA of all Australian isolates studied contained sequence similar to *apxIIA* gene and serovar 1 and 5 isolates also contained sequence similar to *apxIA* gene. Endonucleases producing at least two restriction sites within the *apxIA* or *apxIIA* genes were chosen to digest the *A* pleuropneumoniae DNA (Figures 3C and 4B). When the *A pleuropneumoniae* DNA were digested with *Pst*I and then hybridised with *apxIIA* probe, a 1.9 kb DNA fragment was found in all isolates tested (Figure 3A). When the *A pleuropneumoniae* DNA were digested with *Pst*I and *Sac*I and then hybridised with the *apxIIA* probe, DNA fragments of about 1.5 kb and 0.4 kb were found in all

Figure 3. Southern hybridisation of genomic DNA of Australian isolates of *A pleuropneumoniae* to pJFF727 (plasmid containing gene encoding structural protein of ApxII, *apxIIA*). **A**: Bacterial genomic DNA(~20 mg) digested with *Pst* I. Arrow indicates the 1.9 kb DNA fragments. **B**: Bacterial genomic DNA digested with *Pst* I and *Hin*dIII. Large and small arrows indicate the 1.2 kb and 0.7 kb DNA fragments, respectively. Molecular weight marker (I) is *Hin*dIII- digested I (~1 mg). **C**: Genetic map of *apxIIA* showing relevant restriction sites (Chang *et al* 1989; Frey *et al* 1992).



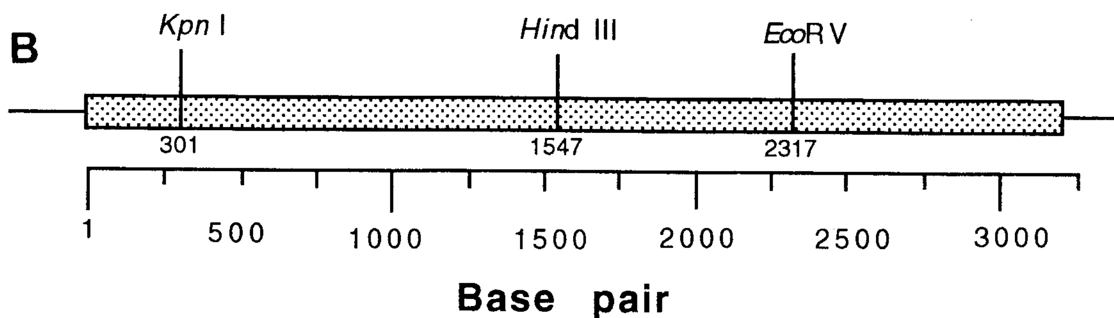




Figure 4. Southern hybridisation of genomic DNA of Australian isolates of *A pleuropneumoniae* to pJFF729 (plasmid containing gene encoding structural protein of ApxI, *apxIA*). **A**: Bacterial genomic DNA (~ 10 mg) digested with *KpnI* and *EcoRV*. Arrows indicate the 2.0 kb DNA fragments. Molecular weight marker (l) is *HindIII*-digested l (~1 mg). **B**: Genetic map of *apxIA* showing relevant restriction sites (Frey *et al* 1991).

isolates (data not shown). Digestion of the *A pleuropneumoniae* DNA with *PstI* and *HindIII* and then hybridisation to the *apxIIA* probe, demonstrated DNA fragments of 1.2 kb and 0.7 kb in all isolates except isolates HS12 of serovar 1 (Figure 3B).

When *A pleuropneumoniae* DNA was digested with *KpnI* and *EcoRV* and then hybridised with *apxIA* probe, a DNA fragment of 2.0 kb was found in all isolates of serovars 1 and 5 (Figure 4A). Digestion of *A pleuropneumoniae* DNA with *KpnI* and *HindIII*, and with *EcoRV* and *HindIII*, and then hybridisation to the *apxIA* probe, demonstrated DNA fragments of about 1.2 kb and 0.8 kb respectively, in serovar 1 and 5 isolates but not in other serovars (data not shown).

In Figures 3 and 4, a number of bands in addition to those described above also appeared in the hybridisation. The presence of those additional bands was interpreted as a consequence of using the whole apxIA or apxIIA as probes and incomplete digestion of the bacterial DNA.

## Discussion

This study characterised haemolytic products and associated structural genetic content of Australian isolates of *A pleuropneumoniae*. Among the 12 known serovars of *A pleuropneumoniae* identified world-wide, 5 serovars, No. 1, 2, 3, 5 and 7, have been isolated in Australia (Eaves and Blackall 1988; Stephens *et al* 1990). We found that culture supernatants of Australian isolates of serovars 1 and 5 were strongly haemolytic, whereas those of serovars 2, 3 and 7 were not, or only weakly, haemolytic, or produced no such activity. Although culture supernatants from serovar 2, 3 and 7 isolates were not, or were haemolytic, the amounts of protein secreted by these isolates into culture were not significantly different from those secreted by serovar 1 or 5 isolates.

On CBA, *A pleuropneumoniae* forms two distinct colony types. The first, which predominates in low passage cultures, is waxy and difficult to remove from the agar plate, whereas the second type,

which predominates in cultures that have been maintained for several passages, is soft and easily removed (Shope 1964). Both colony types have an equal amount of capsular materials and have identical outer membrane protein profiles (Rapp *et al* 1986). However, the waxy colonies of *A pleuropneumoniae* consistently produced lower titres of haemolytic Apx in this study. This may be because of poorer adaptation to in-vitro culture conditions. When incubated in broth media or RPMI-1640, the *A pleuropneumoniae* with waxy colonies grew in floccules. However, when inoculated into pigs, *A pleuropneumoniae* from waxy colonies is as virulent as, or even more virulent than, organisms from soft colonies (Shope *et al* 1964). This evidence suggests that the virulence of an isolate does not always correlate with its capability to produce haemolytic Apx in culture.

The reported characteristics of the haemolysin were very confused before the cloning and sequencing of Apx or *A pleuropneumoniae* haemolysin genes. For example, the haemolysin was described as very stable, carbohydrate (Nakai *et al* 1983) or extremely unstable, RNA-dependent protein (Martin *et al* 1985). The toxin has also been thought to be one of the bacterial thiol-activated toxins because it is inhibited by cholesterol and oxygen (Rosendal *et al* 1988).

After the successful cloning and sequencing of the haemolysin genes (Chang *et al* 1989; Gygi *et al* 1990; Frey *et al* 1991), it has become clear that all serovars, except serovar 10, produce a weak haemolysin (ApxII) and serovars 1, 5, 9, 10 and 11 produce a strong haemolytic factor (ApxI) in addition to ApxII. Based on the organisation and DNA sequence of the *apx* operons, ApxI and ApxII are classified as members of the RTX-toxin family, which is characterised structurally by the presence of glycine-rich nonapeptide repeats in the carboxyl-terminal third of the toxin protein (Chang *et al* 1989; Frey *et al* 1991).

Most studies on the molecular biology of the Apx were performed on reference strains (Frey *et al* 1991; Smits *at al* 1991; Frey *et al* 1992; Jansen *et al* 1992). Since serotyping of *A pleuropneumoniae* is based on the antigenic similarity of the polysaccharide capsule, and so far no correlation between the antigenic type of the capsule and the presence of different Apx genes has been established, isolates of the same serovar may contain different Apx genes. The present study, however, demonstrated that the structural genes encoding the haemolytic Apx in Australian field isolates were similar to those of their respective reference strains.

That the *apxIIA* gene is carried by, and is similar, in all field strains, as shown in Southern blot analysis in this study, could be useful in developing better diagnostic techniques. For example, the 2 kb *PstI* fragment of the *apxIIA* gene may be suitable for a probe for in-situ hybridisation studies to localise the organism in histological tissue sections. Oligonucleotide primers corresponding to the sequence of the *apxIIA* have been shown to specifically amplify *apxIIA* genes from chromosomal DNA of *A pleuropneumoniae* reference strains of serovars 1 and 2 (Frey *et al* 1992). Thus, the polymerase chain reaction using primers based on the published DNA sequence of the *apxIIA* may be a good choice as a rapid and accurate diagnostic tool for disease detection.


## Ackowledgments

We thank Dr J Frey, Institute for Veterinary Bacteriology, University of Berne, Switzerland, for supplying us with the cloned *apxIA* and *apxIIA*. This work was funded by the Pig Research and Development Corporation of Australia.


## References


Cameron RD and Kelly WR (1979) *Aust Vet J* **55**: 389

Chang YF, Young RY and Struck DK (1989) *DNA* **8**: 635

Eaves LE and Blackall PJ (1988) *Aust Vet J* **65**: 379

Eaves LE, Blackall PJ and Fegan M (1989) *Aust Vet J* **66**: 1

Frey J and Nicolet J (1988) *Infect Immun* **56**: 2570

Frey J, Meier R, Gygi D and Nicolet J (1991) *Infect Immun* **59**: 3026

Frey J, van den Bosch H, Segers R and Nicolet J (1992) *Infect Immun* **60**: 1671

Frey J, Bosse JT, Chang YF, Cullen JM, Fenwick B *et al* (1993) *J Gen Microbiol* **139**: 1723

Gygi D, Nicolet J, Frey J, Cross M, Koronakis V and Hughes C (1990) *Mol Microbiol* **4**: 123

Inzana TJ, Todd J, Ma JN and Veit H (1991) *Microb Pathog* **10**: 281

Jansen R, Briaire J, Kamp EM and Smits MA (1992) *Infect Immun* **60**: 630

Johnson JL (1991) In *Nucleic Acid Techniques in Bacterial Systematics*, edited by Stackebrandt E and Goodfellow M, John Wiley, Chichester, p 1

Kamp EM, Popma JK, Anakotta J and Smits MA (1991) *Infect Immun* **59**: 3079

Martin PG, Lachance P and Niven DF (1985) *Can J Microbiol* **31**: 456

Mason RW, McKay RW and Corbould A (1982) *Aust Vet J* **58**: 108

Mylrea PJ, Fraser G, Macqueen P and Lambourne DA (1974) *Aust Vet J* **50**: 255

Nakai T, Sawata A and Kume K (1983) *Am J Vet Res* **44**: 344

Rapp VJ, Munson RJ and Ross RF (1986) *Infect Immun* **52**: 414

Rogers RJ, Eaves LE, Blackall PJ and Truman KF (1990) *Aust Vet J* **67**: 9

Rosendal S, Devenish J, MacInnes JI, Lumsden JH, Watson S and Xun H (1988) *Am J Vet Res* **49**: 1053.

Rosendal S, Mitchell WR, Weber M, Wilson MR and Zaman MR (1980) *Proc Int Pig Vet Soc Congr*, **5**: 221

Sambrook J, Fritsch EF and Maniatis T (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn, Cold Spring Harbor Laboratory Press, Salem

Shope RE (1964) *J Exp Med* **119**: 357

Shope RE, White DC and Leidy G (1964) *J Exp Med* **119**: 369

Smits MA, BriaireJ, Jansen R, Smith HE, Kamp EM and Gielkens AL (1991) *Infect Immun* **59**: 4497

Stephens CP, Gibson JA and Blackall PJ (1990) *Aust Vet J* **67**: 462


*(Accepted for publication 4 April 1995)*