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Assessment of candidate antigens of Actinobacillus pleuropneumoniae for the serodiagnosis of porcine pleuropneumonia

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Assessment of candidate antigens of *Actinobacillus pleuropneumoniae* for the serodiagnosis of porcine pleuropneumonia

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Abstract

A 39 kDa outer membrane protein (OMP) was extracted and purified from *Actinobacillus pleuropneumoniae* serovar 1 (HS54), 7 (WF83) and 12 (HS143). The 39 kDa OMP of all three serovars showed strong immunoreactivity against sera from pigs challenged with *A. pleuropneumoniae* serovars 1, 7 and 12. Furthermore, the 39 kDa OMP from serovar 1, when used as an ELISA antigen showed strong immunoreactivity against sera from pigs that were infected with various *A. pleuropneumoniae* serotypes. This result suggests that the 39 kDa OMP may be used as an ELISA antigen to detect *A. pleuropneumoniae* regardless of the infecting serotype.

Actinobacillus pleuropneumoniae is known to produce three members of the repeat in toxin (RTX) family of toxins, which include, ApxI, ApxII and ApxIII. Recently, a fourth RTX toxin has been identified (ApxIV), cloned, and the N-terminal (N-apxIV) and C-terminal (C-apxIV) domains expressed. We were able to purify and assess the immunoreactivity of the recombinant ApxIV domains. Recombinant C-apxIV was observed to be more immunoreactive than recombinant N-apxIV when used as ELISA antigen. In an ELISA assay involving hemolysin (ApxI) toxin along with the recombinant C-apxIV and N-apxIV, sera from pigs infected with *A. pleuropneumoniae* recognized all three toxins. However, the immunoreactivity was significantly different for the recombinant ApxIV toxin domains compared to that of ApxI. ApxI showed significantly stronger reactivity at all stages of infection. This result suggests that further work is needed to confirm whether the recombinant ApxIV toxin domains can be used as ELISA reagents in the detection of *A. pleuropneumoniae* during the course of an infection.

Declaration

This work has been submitted in accordance with the regulations of the University of Wollongong in partial fulfillment of the degree of Masters (Honours) in Science. To the best of my knowledge, all use of information from various authors and scientific journals, etc. have been acknowledged and duly referenced. Other than that, this work is original and has not been submitted for a degree at any University.

Signed,

Sameer M Dixit

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Abbreviations

ABTS	2,2-azino bis (3 ethylbenzthiazoline-6-sulphonic acid)
amp	ampere
Amp	Ampicillin
APXIVA3C'-His/rC-ApxIV	C-apxIV; carboxy-terminal domain of ApxIV
APXIVAN'a-His/rN-ApxIV	N-apxIV; amino-terminal domain of ApxIV
BHIB	Brain heart infusion broth
ELISA	Enzyme Linked Immunosorbent Assay
g	gravity
IPTG	Isopropyl-β-D-thiogalactopyranoside
kDa	kilo-dalton
LB	Luria Bertani
m	milli
M	molar
μ	micro
Min	minutes
OD	optical density
PBS	phosphate buffered saline
r	resistant
V	volts
Vol/v	volume
w	weight
°C	degrees Celsius

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Chapter 1: Introduction

1.1 Family *Pasteurellaceae*

The family *Pasteurellaceae* includes the genera *Haemophilus*, *Actinobacillus* and *Pasteurella*. The members of this family were initially recognized because of the clinical conditions they caused in host animals (Zinnemann, 1981). They were then recognized on the basis of their morphology, their ecology (host range and disease) and biochemical tests (Mannheim, 1984). Later on, the organisms were assigned to the family *Pasteurellaceae* primarily on their requirement for the growth factors hemin and NAD.

Phenotypic classification was based on numerical taxonomy, which examined large (>150) numbers of strains using a large number of phenotypic tests (>150) and similarities based on the results of these tests were measured. At best, the approach could be used to look at only 10-20% of the genome. The different ways in which similarity co-efficients were utilized to estimate resemblance made it difficult to accurately phenotype these bacteria (Austin and Priest, 1986) In the 1980s, a concerted effort was made to examine the genotypic properties of the members of this family, moving away from the phenotypic characteristics. Some of the current taxonomic methods used to classify *Haemophilus*, *Actinobacillus* and *Pasteurellaceae* (HAP) include chemotaxonomy (analysis of composition of cell walls), protein analysis (polypeptide fingerprinting), %G+C determination, DNA analysis, rRNA analysis and to a lesser extent genetic transformation and analysis of quinones (MacInnes and Borr, 1990).

According to the latest edition of Bergey's Manual of Systemic Bacteriology (1984), there are 27 approved species in the HAP group. There are 16 in the Genus *Haemophilus*, five in the Genus *Actinobacillus*, and six in the Genus *Pasteurella* (table 1) (Mannheim, W., 1984).

Table 1.1. Members of the HAP family (adapted from Bergey's manual, 1984)

Haemophilus	*Actinobacillus*	*Pasteurellaceae*
1. *H. influenzae*	1. *A. lignieresii*	1. *P. multocida*
2. *H. aegyptius*	2. *A. equuli*	2. *P. pneumotropica*
3. *H. haemolyticus*	3. *A. suis*	3. *P. haemolytica*
4. *H. haemoglobinophilus*	4. *A. capsulatus*	4. *P. ureae*
5. *H. ducreyi*	5. *A. actinomycetemcomitans*	5. *P. gallinarum*
6. *H. parainfluenzae*		
7. *H. parahaemolyticus*		
8. *H. paraphrohaemolyticus*		
9. *H. pleuropneumoniae*		
10. *H. paracuniculus*		
11. *H. aphrophilus*		
12. *H. paraphrophilus*		
13. *H. segnis*		
14. *H. parasuis*		
15. *H. parragallinarum*		
16. *H. avium*		
a) *"H. somnus" (sub-categorization of H. avium)*		
b) *"H. agni" (sub-categorization of H. avium)*		
c) *"H. equigenitalis" (sub-categorization of H. avium)*		

1.2 Actinobacillus pleuropneumoniae

Actinobacillus pleuropneumoniae is a facultative, anaerobic, non-motile Gram-negative coccobacillus within the HAP family. *A. pleuropneumoniae* is the etiologic agent of swine pleuropneumonia (Nicolet, 1992) . Before 1983, this organism was known as *Haemophilus pleuropneumoniae*. However, its similarity with *Actinobacillus lignieressie* and not *Haemophilus influenzae* in DNA hybridization studies led to the transfer of this organism to the Genus *Actinobacillus* (Pohl *et al.*, 1983). *A. pleuropneumoniae* has been classified into two biotypes. Biotype 1 strains are nicotamide adenine dinucleotide (NAD) dependent while biotype 2 strains on the other hand, are NAD-independent (Haesebrouck *et al.*, 1997). *A. pleuropneumoniae* is known to have 14 serotypes, based on capsular antigens. There are 12 serotypes within biotype 1 (Nielsen, 1986) and 2 within biotype 2 (Fodor *et al.*, 1989). Serotypes 1 and 5 are further subdivided into 1a and 1b and 5a and 5b respectively (Jolie *et al.*, 1997). The first report of *A. pleuropneumoniae* in Australia was in New South Wales in 1974 (Mylrea *et al.*, 1974). Serovar 1 is most common in Victoria, while serovar 7 is found commonly in New South Wales, Queensland, and Victoria. Serovar 12 has been isolated in New South Wales, Queensland, Victoria, and South Australia (Blackall and Pahoff, 1995).

1.3 Swine pleuropneumonia

Swine pleuropneumonia is a severe lung disease in pigs caused mainly by *A. pleuropneumoniae* (Nicolet and Scholl., 1981). This disease is characterized by necrotizing hemorrhagic bronchopneumonia with accompanying fibrinous pleuritis (Nicolet *et al.*, 1992). Acute pleuropneumonia is characterized by fibrinous adhesion caused by *A. pleuropneumoniae* in the pleural cavity accompanied by necrotic and hemorrhagic lesions in the lungs, leading to death in 24 to 48 hours following infection (Haesebrouck *et al.*, 1997). Acute pleuropneumonia causes high mortality in non-immune pigs. The chronic form of the disease, which does not result in immediate death, does cause economic losses due to poor weight gain by

infected pigs and failure to thrive (Inzana *et al.*, 1991). Due to these reasons, *A. pleuropneumoniae* is considered a threat to the pig industry (Pointon *et al.*, 1995).

1.4 Virulence factors

A. pleuropneumoniae, as well as other pathogenic bacteria in the family *Pasteurellaceae* are generally characterized by their ability to (i) colonize mucosal surfaces (ii) invade host tissues, (iii) survive and multiply in the host, (iv) interfere with host defenses, and eventually (v) damage the host (Nicolet, 1990). The bacterium possesses adhesion structures, capsular polysaccharides, surface structures such as outer membrane proteins (OMPs), lipopolysaccharides (LPS) and secrete extracellular toxin products, among them exotoxins, in order to help the bacterium infect host animals and in many cases, cause disease (Table 1.2).

Not all *A. pleuropneumoniae* serotypes are equally pathogenic. The virulence of *A. pleuropneumoniae* is different across serotypes. In general, biotype 1 is more virulent than biotype 2. Within biotype 1 group, serovars 1a, 1b, 5a, 5b, 9 and 10 are considered most virulent (Haesebrouck *et al.*, 1997).

Table 1.2. Summary of HAP family virulence factors, and immune protection with antibodies (modified from Wannemeuhler *et al.*, 1994).

Cellular component	Role in disease
Capsule	Anti-phagocytic, inhibition of complement activation
Endotoxin	Pyrogenicity, toxicity
Pili	Cell adhesion
Outer membrane proteins (OMPs)	Antigenic variation, nutrient acquisition
Exotoxin	Cell death, fluid secretion
Cytoplasmic membrane and cytosolic proteins	Nutrient acquisition, adherence

1.4.1 Capsule

Within the HAP group, the presence of the polysaccharide capsule is a common feature. The encapsulated pathogens in the HAP family are *H. influenzae*, *A. pleuropneumoniae*, *P. haemolytica* and *P. multocida* (Nicolet, 1990). Bacteria that are encapsulated can be divided into different serotypes based on structural and antigenic differences in the capsule. In many cases, serotypes can be differentiated into subtypes based on differences within somatic antigens (Inzana, 1991).

The capsule surrounds the outer membrane of the cell. These can be observed by light microscopy. Each of the capsules of the *Pasteurellaceae* that have been purified and analyzed are acidic, of high molecular weight and heterogeneous polysaccharides. The polysaccharide capsules are composed of repeating units of two or three sugars or aminosugars. Also present are glycerol, acetyl phosphate, carboxyl and uronic acid groups. Phosphate groups or uronic acid provide the negative charge on the capsule (Inzana, 1991).

Capsule is a required virulence factor in *A. pleuropneumoniae* pathogenesis because its presence prevents opsonization and clearance of bacteria in the lungs of the host (Inzana, 1991). Capsular polysaccharides can inhibit phagocytosis, mask underlying antigens from antibodies and prevent activation of complement (Wannemuehler *et al.*, 1994). Although the actual mechanism of resistance to killing by antibodies is not very clear, indications are that encapsulation of the pathogen interferes with deposition of the membrane attack complex of complement.

The serotype specificity of *A. pleuropneumoniae* is due to the presence of capsular antigens (Fedorka-Cray *et al.*, 1994). The differences in virulence between different serotypes or even within the same serotype have also been attributed to

the structure of the capsule of the *A. pleuropneumoniae* serotypes (Jensen *et al.*, 1986).

1.4.2 Adhesins

The ability of most pathogenic bacteria to induce disease depends in their ability to adhere to epithelial cells. During the initial step of pathogenesis, bacteria colonize host sites by engaging their surface bound adhesins with cognate receptors available on host cells. This recognition and attachment process is considered to be the first steps in establishing bacteria at a given site. It has been proposed that *A. pleuropneumoniae* adheres to lower respiratory epithelial cells, possibly leading to a local high concentration of Apx toxins at the surface of those eukaryotic cells. Haemagglutination as well as *in vitro* adherence of the pathogen to porcine tracheal epithelial cells, frozen lung cells, and mucus of the respiratory tract have also been reported (Haesebrouck *et al.*, 1997). The role for fimbrae in the adhesion of *A. pleuropneumoniae* has not been demonstrated although they have been detected in this organism (Dom *et al.*, 1994).

1.4.3 Outer membrane proteins (OMPs)

Outer membrane proteins are considered to be important in regulation of surface proteins in response to environmental growth conditions. Across the HAP family, OMPs seem to play various roles in pathogenicity of the bacteria. Some of these proteins have been found to induce the synthesis of protective serum antibodies to *P. haemolytica* (Mosier *et al.*, 1989), as well *as A. pleuropneumoniae* (MacInnes and Rosendal, 1987). The best-characterized OMPs with immunoprotective properties are the P2 and P6 porins of *H. influenzae* and the protein H of *P. multicoda*. Although the exact biological mechanisms of these proteins are not known, evidence of the participation of OMPs in virulence is furnished by an OMP with antiphagocytic activity in *P. multicoda*. *A. pleuropneumoniae* is able to synthesize IROMPS (iron regulated OMPs) with receptor functions for complexed

iron, indicating the role of OMPs in the adaptability of the bacterial cell to the host environment (Deneer *et al.*, 1989).

Certain outer membrane proteins have been identified in *A. pleuropneumoniae* which are conserved across serovars, unlike serotype dependent lipopolysaccharides which are the preferred antigens in *A. pleuropneumoniae* detection systems. All serovars express several common outer membrane proteins, including the peptidoglycan associated lipoprotein PalA (14 kDa), a protein that varies from 38-42 kDa and a 48 kDa protein (Frey *et al.*, 1996). Immunologically, PalA, the 32 kDa and the 39 kDa protein are found to be immunodominant, based on immunoreactivity studies (Frey *et al.*, 1996; Jansen, 1994; MacInnes and Rosendal, 1987). It has been shown that convalescent phase sera from *A. pleuropneumoniae* infected pigs contain antibodies against these proteins and some other OMPs (Cruz *et al.*, 1996). Some of the immunodominant OMPs of *A. pleuropneumoniae* have been cloned and expressed to be used as potential antigens for vaccine/diagnostic purposes. These include the 48 kDa OMP of serotype1 and serotype 5, and a 16 kDa OMP of serotype 5 (Gerlach *et al.*, 1993; Bunka *et al.*, 1995; Cruz *et al.*, 1996).

1.4.4 Lipopolysaccharides (LPS)

LPS are high molecular weight molecules that form part of the outer membranes of Gram-negative bacteria. LPS are comprised of a complex lipid material and are involved in the release of proinflammatory mediators that activate various cells, including macrophages and neutrophils. In the HAP group of bacteria, LPS have been implicated as major factors in the pathogenesis of disease caused by members of this group. This is due to its prominence as a surface component of the pathogen and ability to cause tissue damage.

Structurally, liposaccharides are high molecular weight cornplex macromolecules,

which are an integral component of the outer membrane of Gram-negative bacteria. Although the basic structure of LPS is similar in all Gram-negative bacteria, there are variations in terms of outer saccharides. The polysaccharide portion of the LPS is divided into two regions. The outer region (O-antigen) is comprised of repeating oligosaccharide chains of various lengths. It is the vast number of potential constituents and variable linkages that provide the O- antigens with antigenic specificity. While capsular polysaccharides form the basis of the serotype differentiation of HAP group bacteria, O-antigens are important in some cases (Fenwick, 1990). The inner region of LPS is less variable both structurally and antigenically.

The *Haemophilus* species appear to be unique among the HAP group of bacteria in that their liposaccharides lack polymerized O-antigen. The term lipooligosaccharides has become standard when referring to the liposaccharides of *Haemophilus* species. The structural, and thus antigenic, variability of the saccharide portion of the LPS, probably allows it to play a role in helping the bacterium avoid antigen-specific immune responses. The major contribution of LPS to virulence is its ability to activate non-specific host defenses. The ability of LPS to trigger host defence system rests in the lipid portion of the molecule. Lipopolysaccharides may work together with exotoxins in mediating the disease process (Fedorka-Cray *et al.*, 1994). It has been reported that purified *A. pleuropneumoniae* LPS has the potential to cause tissue damage. In that study, the lesions induced by *A. pleuropneumoniae* LPS in pig lungs were neither haemmorhagic nor necrotic, indicating that LPS is not responsible for the typical *A.pleuropneumoniae* lesions. It may, however, contribute to the formation of such lesions (Haesebrouck *et al.*, 1997).

1.4.5 Exotoxins

Exotoxins are extracellular soluble proteins actively released by live organisms that play a major role in evasion of the hosts' first line of defence. The repeat in toxin

(RTX) family of toxins, when present in high concentrations, form pores in membranes of phagocytic and other target cells resulting in osmotic swelling and cell death. They might also be involved in development of lesions in lungs, toxicity for endothelial cells and provoke an oxidative burst in macrophages and neutrophils, resulting in excessive production of oxygen radicals which can have deleterious effects on host cells (Haesebrouck *et al.*, 1997).

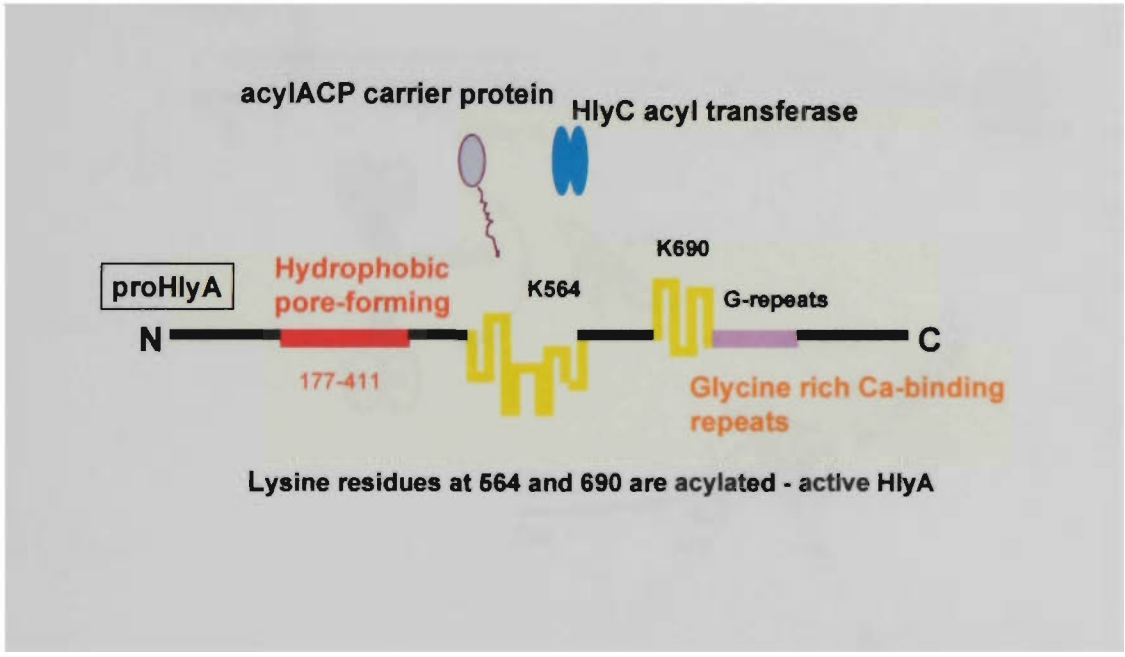


Fig 1.1. Basic structure of ApxI (hemolysin) RTX toxin. Toxins of the RTX family contain a hydrophobic pore forming region in the N terminus (in red) , a glycine repeat region in the C terminus (purple) and a middle region containing lysine residues (yellow). Activation of the toxin involves acylation of the lysine residues at positions 564 and 690 by co-synthesized HlyC which is a homodimeric putative acyltransferase that uses acyl-acyl carrier protein (acyl-ACP) as the fatty acid donor to the lysine residues (Stanley, 1994).

In *A. pleuropneumoniae*, three protein toxins have been identified, which fall in the category of RTX family of toxins. These possess hemolytic and neutrophil toxic (cytotoxic) activity (Frey, 1995). RTX toxin family is distinguished by a strongly hydrophobic domain in the N terminal, which is involved in the insertion of the toxin molecule into the target host cell (fig. 1.1). There is a glycine repeat region in the

C-terminal which is believed to be involved in calcium binding required for target cell recognition and binding. Lysine residues in the middle region of the toxin operon are considered important in the activation of the toxin. Acylation of the lysine residues by acyl transferase activates inactive prohemolysin into active hemolysin (Stanley, 1998) (fig. 1.2). RTX family of toxins includes hemolysin from *E. coli*, leukotoxins of *Pasteurella* and *Actinobacillus*, and the adenylate cyclase-hemolysin of *Bordetella pertussis*.

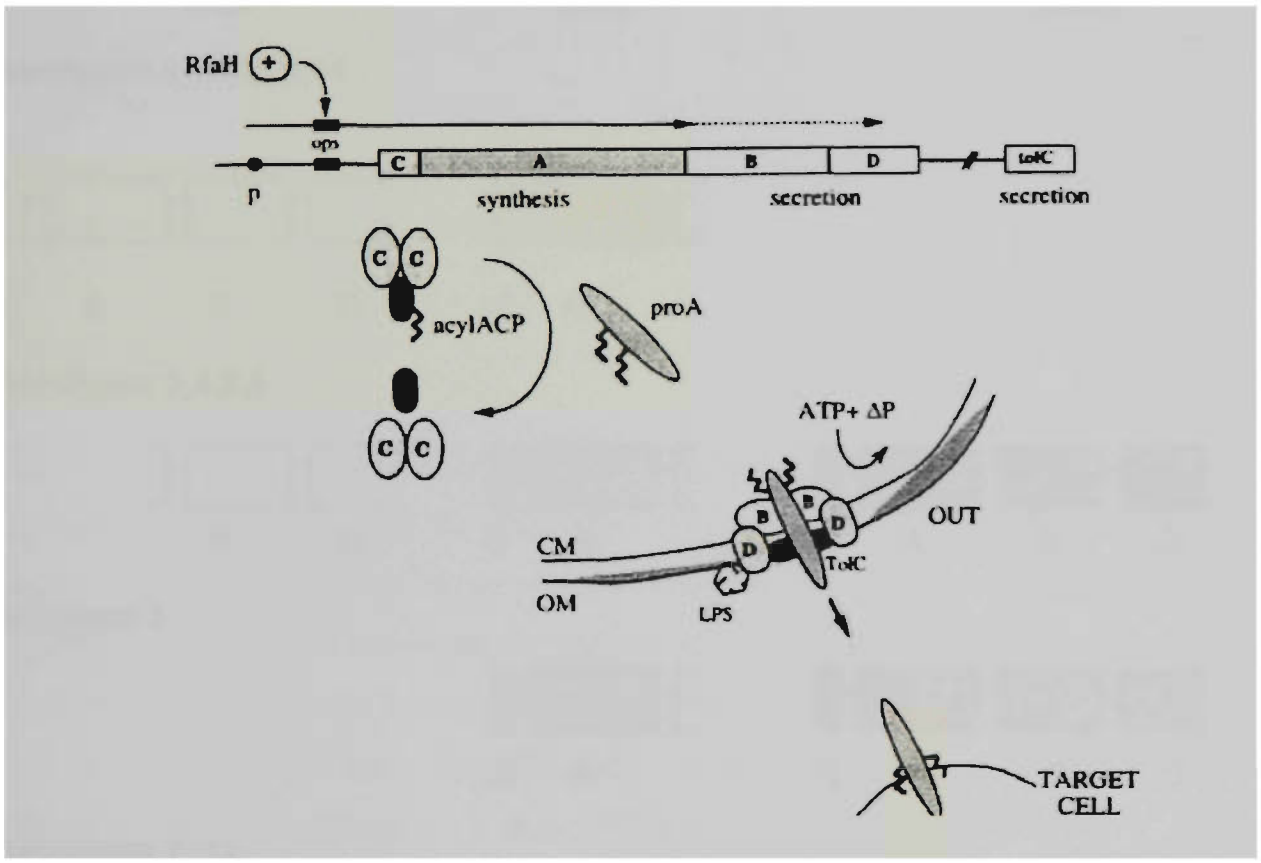


Fig. 1.2. Hemolysin synthesis, maturation, and export by *E. coli* and other related Gram-negative bacteria including *A. pleuropneumoniae*. The hemolysin gene encodes inactive pro-hemolysin (proA), which is activated by HlyC with the help of carrier protein (acylACP). (CM, cytoplasmic membrane; OM, outer membrane; acylACP, acyl- Acyl Carrier Protein; RfaH, elongation protein [adapted from Stanley *et al.*, 1998].

A. pleuropneumoniae produces three toxins, designated ApxI, ApxII and ApxIII. Nucleotide sequence analysis of the ApxI-, ApxII-, and ApxIII-coding genes have demonstrated that these toxins are closely related members of the RTX toxin family. RTX toxins are encoded by operons that consist of four contiguous genes,

C, A, B, and D. The A gene encodes the structural toxin that is activated by the C gene encoded protein. The B and D gene-encoded proteins are involved in the secretion of the RTX-toxins (Holland *et al.*, 1990). All three Apx toxins strongly resemble each other but differ in size, function and presence in *A. pleuropneumoniae*. Not all serotypes of *A. pleuropneumoniae* produce the same combination of the toxins, and spontaneous loss of toxin production is known to occur (Anderson *et al.*, 1991).

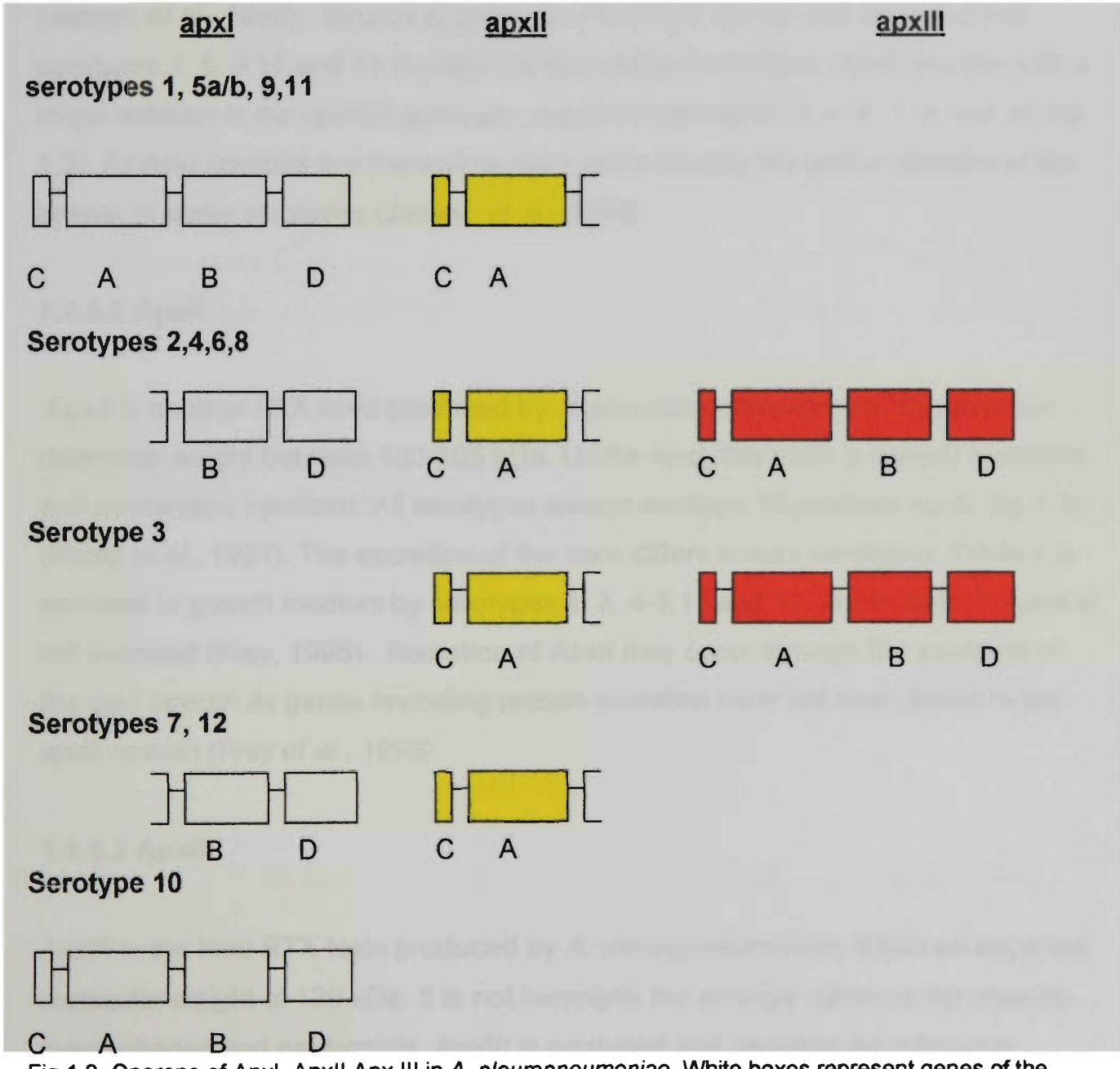


Fig 1.3. Operons of ApxI, ApxII Apx III in *A. pleuropneumoniae*. White boxes represent genes of the apxI operon; Yellow boxes represent genes of the apxII operon; red boxes represent genes of the apxIII operon. Half boxes represent 5'- terminal truncated apxIA (white) or 3'- terminal truncated apxIB (yellow) genes [adapted from Beck *et al.*, 1994].

1.4.5.1 ApxI

ApxI, is a RTX toxin with an apparent molecular mass of 105 kDa to 110 kDa. It is considered strongly hemolytic and strongly cytotoxic to pig alveolar macrophages and neutrophils. ApxI is produced and secreted by serotypes 1, 5a, 5b, 9, 10 and 11 (Beck *et al.*, 1994). However, sequences homologous to the secretion genes apxIBD of the ApxI operon are present in all 12 serotypes except serotype 3 (Jansen *et al.*, 1993). Structural analysis of the ApxI operon has revealed that serotypes 1, 5, 9,10 and 11 contain the four genes apxICABD. ApxI operons with a major deletion in the apxICA gene are present in serotypes 2, 4, 6, 7, 8 and 12 (fig. 1.3). All ApxI operons are transcriptionally active despite the partial deletion of the operon in some serotypes (Jansen *et al.*, 1993).

1.4.5.2 ApxII

ApxII is another RTX toxin produced by *A. pleuropneumoniae* with an apparent molecular weight between 103-105 kDa. Unlike ApxI, this toxin is weakly hemolytic and moderately cytotoxic. All serotypes except serotype 10 produce ApxII (fig.1.3) (Kamp *et al.*, 1991). The secretion of the toxin differs across serotypes. While it is secreted in growth medium by serotypes 1, 2, 4-9,11 and 12, in serotype 3, ApxII is not secreted (Frey, 1995). Secretion of ApxII may occur through the products of the apxI operon as genes encoding protein secretion have not been found in the apxII operon (Frey *et al.*, 1993).

1.4.5.3 ApxIII

ApxIII is the third RTX toxin produced by *A. pleuropneumoniae*. It has an apparent molecular weight of 120 kDa. It is not hemolytic but strongly cytotoxic for alveolar macrophages and neutrophils. ApxIII is produced and secreted by reference strains of serotype 2, 3, 4, 6 and 8 (Kamp *et al.*, 1991). Sequence analysis of ApxIIICABD genes of all serotypes reveals very few serotype specific differences.

Only the C-terminus of ApxIIIA of serotype 2 differs from ApxIIIA of the other serotypes (Jansen *et al.*, 1994). Those differences are located between the glycine-rich repeats and the secretion signal.

1.4.5.4 ApxIV

Recently a fourth RTX toxin determinant has been characterized (Schaller et al., 1999). Anderson and McGuinnes (1997) reported the identification and cloning of a lacZ analogue in *A. pleuropneumoniae.* Sequence information revealed the presence of a RTX toxin downstream. Using this information, Schaller and colleagues designed primers and cloned this toxin, designating it ApxIV. The function of the toxin has not been established yet. ApxIV is claimed by Schaller and colleagues (1999) to be expressed by *A. pleuropneumoniae* in pigs during the course of infection only and has not been expressed *in vitro* to date (Table 1.3).

Table 1.3. Summary of Apx toxins of *A. pleuropneumoniae*

RTX toxin	MW size	Function	Serovars	Reference
ApxI	105 kDa	Strongly hemolytic; Strongly cytotoxic	1,5,9,10,11	Beck *et al*, 1994
ApxII	105 kDa	Weakly hemolytic; Moderately cytotoxic	All except 10	Kamp *et al*, 1991
ApxIII	120 kDa	Non hemolytic; Strongly cytotoxic	2,3,4,6,8	Macdonald *et al.*, 1992
ApxIV	202 kDa (predicted)	unknown	All serovars	Schaller *et al.*, 1999

1.5 Diagnosis of pleuropneumonia

1.5.1 Clinical and pathologic diagnosis

A typical outbreak of pleuropneumonia caused by *A. pleuropneumoniae* can have a morbidity of 50% or higher, with 1%-10% mortality. Young pigs are usually the most affected, and the first signs of the disease include stiffness, lameness, anorexia, lethargy, and fever. Clinical signs of a respiratory nature develop soon after, and are characterized by shallow inspiratory volumes, deep non-productive cough as well as marked abdominal pushes.

According to Nicolet and Scholl (1981), swine pleuropneumonia can be classified into four stages. The initial stages include the paracute and acute stages. During the paracute stage of the disease, pigs become very ill with 106.7°C fever, and showing apathy and anorexia, followed by a short period of diarrhea and vomiting. Death occurs within 24-36 hours. The acute stage is characterized by a rise in the body temperature to 105°C-106°C, with pigs showing signs of depression and refusing to eat. Respiratory symptoms with dysprea, coughing and sometimes mouth-breathing are evident. The course of disease can differ within animals, leading to the next stages of subacute, acute or resulting in death. The subacute and chronic symptoms follow after the disappearance of the acute symptoms. Clinical signs include absence of fever, spontaneous or intermittent cough, loss of appetite, and therefore decrease weight gain. Abortions by pregnant sows have been observed in some chronic cases.

It has been suggested that clinical signs are not good indicators of the amount of lung damage (Straw *et al.*, 1990). Due to the rapid progression of this disease, a quick, accurate diagnosis is required in order to initiate treatment.

Diagnosing an acute outbreak of pleuropneumonia is much easier than diagnosing subclinical disease. Slaughter checks are one way to diagnose the infection but in

cases where acute lesions are absent, this type of diagnosis is useless, as slaughter checks won't yield any results. In many cases, even the severe pulmonary lesions induced by *A. pleuropneumoniae* resolve in a few weeks (Noyes *et al.*, 1990).

1.5.2 Microbiological diagnosis

The HAP family of bacteria of veterinary importance has certain similar attributes, and includes the types of disease they cause, routes of transmission, virulence factors and antigens believed to be responsible for providing protective immunity. In the case of *A. pleuropneumoniae*, accurate identification is possible by culture on blood agar and the evidence of small waxy to mucoid colonies that satellite around a Staphylococci cross–streak. Incubation in 5 to 7% concentration of CO_2 aids in initial isolation. A positive urease reaction is an important clue in identifying *A. pleuropneumoniae*. However, biotype 2 which does not require NAD and some strains that are urease negative, can lead to misidentification of this pathogen (Frank *et al.*, 1992; Blanchard *et al.*, 1993). Technologies such as the use of specific DNA probes and polymerase chain reaction provide powerful new methods of confirming an isolate's identity (Sirois *et al.*, 1991).

1.5.3 Serological diagnosis

Over the years, numerous serologic tests have been developed, some of which are currently in use. The complement fixation (CF) test has been the serodiagnosis standard, but during the past few years, its value has been questioned. The reason for this is because the CF test relies heavily on IgM antibodies and as the time between infection and testing increases, the test becomes less reliable. Also, since CF tests detect antibodies to surface antigens, they may fail to detect pigs infected with untypable *A. pleuropneumoniae* strains. As a result, CF tests have few false-

positive results, but high false-negative results compared to other serological tests. One other disadvantage of the test is that this test cannot resolve the problem of pro-complementary and anti-complementary activity of some swine sera (Gottschalk, 1994). Even then, CF tests have been valuable in porcine pleuropneumonia control programs. The fact is that even a few CF positive test results are taken as evidence that the herd is infected with *A. pleuropneumoniae* (Fenwick, 1990).

Enzyme Linked Immunosorbent Assay (ELISA) is used to detect antibodies against *A. pleuropneumoniae*. The most common ELISA tests are based on either direct interaction of pig sera with *A. pleuropneumoniae* antigens or on the inhibition of polyclonal rabbit sera by pig sera samples. Most common antigens include LPS and capsular polysaccharide (CPS), which are again, serovar specific. Antibodies against these can have cross reactivity problems with LPS and CPS from other bacterial strains such as *E. coli* in an indirect ELISA due to overlapping epitopes. Subsequently, the chances of cross reactivity may be greater when polyclonal antibodies are used in an inhibition ELISA or if LPS/CPS are the antigens in an indirect ELISA. In contrast to CF tests, ELISA tends to overestimate the prevalence of the disease due to a higher frequency of false positive results.

Cytotoxin neutralization tests detect resistance to important virulence factors and current vaccines do not affect results obtained using this method. Vaccines can cause pigs to test positive by complement fixation testing as well as ELISA. The disadvantage of cytotoxin neutralization tests is that they are complicated to perform and are more expensive (Fenwick, 1990).

1.6 Purpose of the research project

Actinobacillus pleuropneumoniae is the etiologic agent of porcine pleuropneumonia. The presence of this disease, whether in the chronic form or in the acute form, causes major loss to the pig industry. In Australia, the acute form of

this disease has resulted in major losses to the pig industry. Swine pleuropneumonia continues to be a problem in Australia and elsewhere. One of the reasons for this is because there are no tests available that use a "common" antigen that can be used in the detection of *A. pleuropneumoniae*, regardless of the serovar, during the course of an infection.

Early detection of *A. pleuropneumoniae* in pigs is of vital importance in controlling this disease. Pigs carrying *A. pleuropneumoniae* do not necessarily show physical signs of the disease, and thus can intermingle with healthy pigs in the herd and transmit the disease. Early detection would allow for segregation of infected pigs from the herd, protecting other healthy pigs. As mentioned previously, tests that are available for early detection of *A. pleuropneumoniae* rely mostly on serovar specific antigens of the pathogen, such as capsular polysaccharides and lipopolysaccharides. Those tests are good for detecting a *particular A. pleuropneumoniae* serovar, but the presence of 12 serovars of this pathogen makes such tests unreliable. It is therefore important that a new detection system be formulated, one that uses a common antigen, thereby facilitating the detection *A. pleuropneumoniae* regardless of serovar.

Chapter 2: Materials and methods

2.1 Recovery of bacteria from frozen stocks

Escherichia coli and *Actinobacillus pleuropneumoniae* serovars were recovered from frozen 25% glycerol stocks by streaking chipped fragments directly onto chocolate agar plates supplemented with 20 μg/ml NAD (Sigma) (*A. pleuropneumoniae*) or Luria Bertani agar plates supplemented with 100 μg/ml ampicillin (Sigma) (*E. coli*). All plate cultures were incubated at 37°C, *A. pleuropneumoniae* was grown in the presence of 5% (v/v) CO_2.

2.2 Propagation of bacteria in liquid culture

E. coli:

E. coli was grown on Luria Bertani broth supplemented with 100 μg/ml ampicillin. Single colonies were inoculated into 50 ml starter cultures in a 250 ml Erlenmyer flasks and grown overnight on a rotatory shaker (Gallenkamp, 160 rpm). Large scale growth was achieved by adding 10 ml of starter culture into a 300 ml LB broth supplemented with 100 μg/ml ampicillin and monitoring growth over a period of time leading to the log phase and subsequently to lag phase.

A. pleuropneumoniae:

A. pleuropneumoniae strains were propagated in brain heart infusion broth (BHIB) (Oxoid) supplemented with NAD (20 μg/ml, Sigma). Single colonies were inoculated into 50 ml of starter cultures in 250 ml Erlenmyer flasks and grown overnight on a rotatory shaker (Gallenkamp, 100 rpm). Large-scale growth was achieved by adding 10 ml starter culture into a 300 ml BHIB broth supplemented with 20 μg/ml NAD and monitoring growth over a period of time.

2.3 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were electrophoretically separated using a mini Hoeffer gel system under reducing conditions as described by Laemmli (1970). Briefly, 10 mg pelleted whole cell samples were solubilized in Laemmli reducing mixture (5% w/v β-mercaptoethanol; 62.5 mM Tris-Cl pH 6.8; 3% w/v SDS; 10% w/v glycerol). Bromophenol blue tracking dye (final concentration 2%) was added to the sample. The samples were boiled at 100°C for 5 minutes. They were then loaded onto a 8% (*E. coli*) or 10% and12% (*A. pleuropneumoniae*) (w/v) acrylamide running gel with a 4% (w/v) stacking gel. Electrophoresis was carried out at constant a 130 V until the Bromophenol blue tracking dye had electrophoresed to the bottom of the gel.

2.4 Immunoblot analysis

Proteins were electrophoretically transferred from the acrylamide gel to nitrocellulose in a BioRad Trans-Blot apparatus (BioRad Laboratories, Richmond, Ca, USA) under essential conditions described by Towbin *et al.* (1979) except that the transfer was carried out at 30 V overnight rather than 60 V for one hour. Briefly, immunoblots were blocked by incubation in 2% skim milk–Tris saline-TWEEN (TS-TWEEN; 2% w/v skim milk; 20 mM Tris-Cl, pH 7.4; 0.015 M NaCl and 0.01% v/v Tween 20) for 1 hour and washed with TS-Tween for 5 minutes. The nitrocellulose containing the proteins was then reacted for 90 minutes with pig sera diluted 1:500 in TS-Tween. The immunoblot was then washed for 15 minutes, with TS-Tween changed every 5 minutes. Immunoblots were then reacted with horseradish peroxidase-conjugated goat anti-swine IgG (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) for 90 minutes. The immunoblots were then washed for 15 minutes with a TS-Tween change every 5 minutes. The immunoblots were then washed for 5 minutes in freshly prepared 50 mM Tris-HCl buffer (pH 7.6) and the immunoreactive protein bands developed by adding 50 µl freshly prepared

substrate (50 mM Tris-Cl, pH 7.6; 0.12 mM 3,3-diaminobenzidine; 0.03% v/v H_2O_2). The reaction was stopped by washing immunoblots in milliQ water.

2.5 Enzyme Linked Immunosorbent Assay (ELISA)

Antigens obtained from PAGE-PREP fractionation were diluted to 2 μg/ml final concentration in 50 mM bicarbonate buffer (pH 9.6) and 50 μl dispensed into each well of a 96 well plate (Linbro). The plate was then incubated at 4°C overnight. The plates were then washed with milliQ water. Individual sera were diluted 1/500 in TS-Tween 20 and dispensed into the washed plates in a volume of 50 μl. The plates were incubated at 37°C for 90 minutes. The plates were then washed with PBS-Tween (phosphate buffered saline containing Tween 20 at 0.05% w/v) and milli Q water. Goat anti-pig IgG (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) conjugated to horseradish peroxidase, diluted 1/1000 in TS-Tween, was added to the wells in a volume of 50 μl and incubated for 90 minutes at 37°C. The plates were again washed with PBS-Tween and milli Q water. After washing, the binding of the antibodies was confirmed by adding 50 μl 2,2-azino bis (3 ethylbenzthiazoline-6-sulphonic acid) diammonium salt (ABTS) (1 mM ABTS dissolved in citrate phosphate buffer pH 4.2). The plates were then incubated at room temperature on a bench-top shaker and the absorbance read at 414 nm using a Titertek ELISA plate reader.

2.6 Statistics

Student's two-tailed t-Test assuming equal variance was performed (Microsoft Excel, V97, Microsoft Corporation, Ca) to test the correlation between two sets of data. The results were taken as being significant if the probability of Student's t-Test was less than 0.05.

Chapter 3: Evaluation of a 39 kDa outer membrane protein (OMP) of *A. pleuropneumoniae* as a diagnostic ELISA reagent

3.1 Introduction

Outer membrane proteins of *A. pleuropneumoniae* are considered important virulence factors since they are major targets for humoral immune responses in swine infected with the pathogen (Lu *et al.*, 1988).

The major OMPs of *A. pleuropneumoniae* are conserved across all 12 serovars. These include the 16-18 kDa, a 29-32 kDa, a 38-42 kDa and a 48 kDa OMP (Cruz *et al.*, 1996; Rapp *et al.*, 1986; Thwaites *et al.*, 1991). A 76 kDa OMP has been reported to be a conserved as well (Thwaites *et al.*, 1991). Outer membrane proteins of various Gram-negative bacteria have been purified. These include the OMPs of *Pseudomonas maltophila* by the chloroform-methanol method (Chin and Dai, 1989), membrane proteins of *Brucella ovis* (Chin and Turner, 1990), as well as OMPs of *Pseudomonas aeruginosa* by sarkosyl lauroyl sarcosinate (SLS) (Chin *et al.*, 1995). OMPs of *A. pleuropneumoniae* have also been extracted by several groups of researchers (Hartmann *et al.*, 1995; Rapp *et al.*, 1986; Thwaites *et al.*, 1991). The method predominantly used for extraction of membrane proteins as outlined by Chin and Turner (1990) adopts the following protocols: cell breakage to release proteins, high speed centrifugation to separate total membrane vesicles (TMV) containing inner (IMV) and outer membrane vesicles (OMV) from the cell homogenate, extraction of inner membrane protein (IMP) from inner membrane vesicle (IMV) using sodium salicylate (SLS) to solubilize the IMV, and high speed centrifugation on a sucrose pad to separate OMVs from IMPs. The actual process is described in methods and materials section.

The knowledge that some OMPs are immunodominant and are conserved across *A. pleuropneumoniae* serovars (Frey *et al.*, 1996; Jansen, 1994; MacInnes and Rosendal, 1987) leads to the possibility of using those OMPs as ELISA antigens in

the detection of *A. pleuropneumoniae*. The current ELISA antigens are serovar specific as they usually rely on capsular polysaccharides or lipopolysaccharides (Gottschalk *et al.*, 1994; Bosse *et al.*, 1990; Radacovici *et al.*, 1994). Lipopolysaccharides are unique for each serotype, due to differences in the side O chain in terms of its length, composition and structure. The use of LPS in ELISA assays is therefore limited to a particular serotype. Serovar specificity is not helpful when any one of the 12 serotypes can be present during the course of an infection. The use of ELISA for detecting *A. pleuropneumoniae* regardless of the serotype would be enhanced greatly if a common *A. pleuropneumoniae* antigen were used as an ELISA antigen. Possible use of OMPs of *A. pleuropneumoniae* as common ELISA antigens for the detection of swine pleuropneumoniae was therefore an important topic of research.

3.2 Materials and methods

Only specific materials and methods pertaining to this chapter other than general materials and methods are outlined in this section.

3.2.1 Extraction of outer membrane proteins (OMPs)

Methods outlined by Chin and Turner (1990) were adopted for the purposes of OMP extractions. Bacteria were propagated in broth culture as outlined in the Materials and Method section (Chapter 2). Bacterial cells were killed by addition of 0.5% v/v phenol to the growth culture and incubation at 4°C for 1 hour, after which the killed cells were French pressed (4 passes, 8000 psi) (Chin and Scully, 1986) and centrifuged (15,000g, 10 min, 4°C). The pellet was discarded and the supernatant centrifuged again (15,000g, 15 min, 4°C). The resulting supernatant was then layered onto a sucrose pad (20% sucrose/50 mM HEPES) and centrifuged (150,000g, 90 min, 4°C) (Chin and Dai, 1990). The pellet (TMV or total membrane vesicles) containing both inner and outer membrane vesicles (IMV and OMV) was then rinsed with 50 mM HEPES and resuspended in 2% SLS/50 mM HEPES and rotated overnight (33 rpm) at room temperature (Filip *et al.*, 1973).

This step selectively extracted the inner membrane proteins from the inner membrane vesicle leaving behind the outer membrane vesicles intact. The preparation was then loaded onto a sucrose gradient consisting of discrete layers of 60, 40 and 20% (w/v) sucrose. Following centrifugation at 150,000g for 90 min at 4°C, the outer membrane vesicles were located at the 60/40% (w/v) interphase while inner membrane proteins remained above the 20% (w/v) sucrose layer.

3.2.2 Preparative SDS-PAGE

Preparative SDS-PAGE (PAGE-PREP) technology was used to purify soluble antigen of known molecular weight from whole cell preparations. The BIO-RAD Prep Cell (Model 491) apparatus assembly, gel preparation, and use were performed as per manufacturer's instructions.

A. pleuropneumoniae cells (100 mg) were reduced in 1 ml Laemmli reducing mixture [(5% β-Mercaptoethanol (w/v); 62.5 mM Tris-Cl pH 6.8; 3% SDS; 10% glycerol (w/v)] by boiling (Laemmli, 1970), and several crystals of bromophenol blue were added to dye the sample. The cell preparation was loaded onto a 9% polyacrylamide gel column (Bioprep 491 cell, Bio-Rad), 9 cm in height with a 1 cm stacking gel consisting of 4% polyacrylamide. Columns were run at 40 mA constant current (150-300 V) for 11 hours and fractions (9 ml) were collected immediately after elution of the bromophenol dye. The electrophoresis and elution buffers comprised SDS-PAGE buffer (0.1% SDS, 50 mM Tris, 384 mM glycine), and the flow rate was 1 ml min^{-1}.

Fractions obtained from PAGE-PREP were analyzed by running minigel SDS-PAGE (8 x10 cm, 1.5 mM thickness) consisting of a 10% separating gel and 4% stacking gel. 4 X Laemmli reducing mix was added to part of every second sample to give a final dilution of 1X. The fractions were boiled, and 30 µl samples were then loaded onto the minigel and run (130 V constant voltage) until the

bromophenol dye reached the bottom of the gel. The gels were then stained with Rapid silver staining method (Bloom et al., 1987).

3.2.3 Production of pig antisera

Reference antisera against type strains of *Actinobacillus pleuropneumoniae* representing serovars 1, 7 and 12 were generated by vaccinating pigs (n=3) with $3x10^6$ CFU bacteria formulated in Emulsigen (Intervet/Ausvac/Australia). Pigs were vaccinated on three occasions 4 weeks apart and blood was collected 4 weeks after the last immunization.

To simulate infection regimes, three groups of 3 pigs were each challenged intranasally (IN) with $5x10^9$ CFU (in a volume of 1 ml saline) bacteria representing one of the three *Actinobacillus pleuropneumoniae* type strains. The IN challenged pigs were then allowed to mingle with uninfected cohort pigs (n=6) per group and blood was collected at weekly intervals for 6 weeks from these animals. Pigs used in each of the three experimental groups were confined to separate rooms to ensure that there was no cross-lateral transmission of representative *Actinobacillus pleuropneumoniae* serovars.

3.3 Results

3.3.1 SDS-PAGE analysis of three of the most commonly isolated serovars of *A. pleuropneumoniae* in Australia

Most of the commonly isolated strains of *A. pleuropneumoniae* in Australia belong to serovars 1, 7 and 12 (Blackall and Pahoff 1995). Type strains 4074, WF83 and 1096, selected to represent serovars 1, 7 and 12 respectively, were analyzed by SDS-PAGE under denaturing conditions in an effort to compare the polypeptide antigenic profile. Fig 3.1 shows the presence of at least six intensely stained bands corresponding to molecular sizes of 28 kDa, 30 kDa, 39 kDa, 48 kDa, 67 kDa and 85 kDa in all three serovars. The other minor bands that were present in the three serovars included those resembling a 33 kDa, a 35 kDa, a 37 kDa, a 42 kDa, a 55 kDa, a 60 kDa, a 70 kDa as well as a 93 kDa band.

3.3.2 Immunoreactivity of polypeptide antigens of 3 type strains representing serovars 1 (HS54), 7 (WF83) and 12 (HS143)

The immunoreactivity of polypeptide antigens of serovars 1, 7 and 12 were analyzed by immunoblotting. Whole cell bacteria from each type strain was denatured, electrophoresced by SDS-PAGE and transferred to nitrocellulose membrane. Fig 3.2 shows the immunoreactivity of the 3 type strains following reaction with pooled serum from pigs that had been hyper-vaccinated against HS54 (Sv1, panel A) or WF83 (Sv 7, panel B) or HS143 (Sv 12, panel C). The most prominent immunoreactive bands had apparent molecular sizes of 22 kDa, 30 kDa, 39 kDa and 67 kDa. These bands were consistently found to be immunoreactive for all 3 type strains against sera generated against each of the 3 type strains.

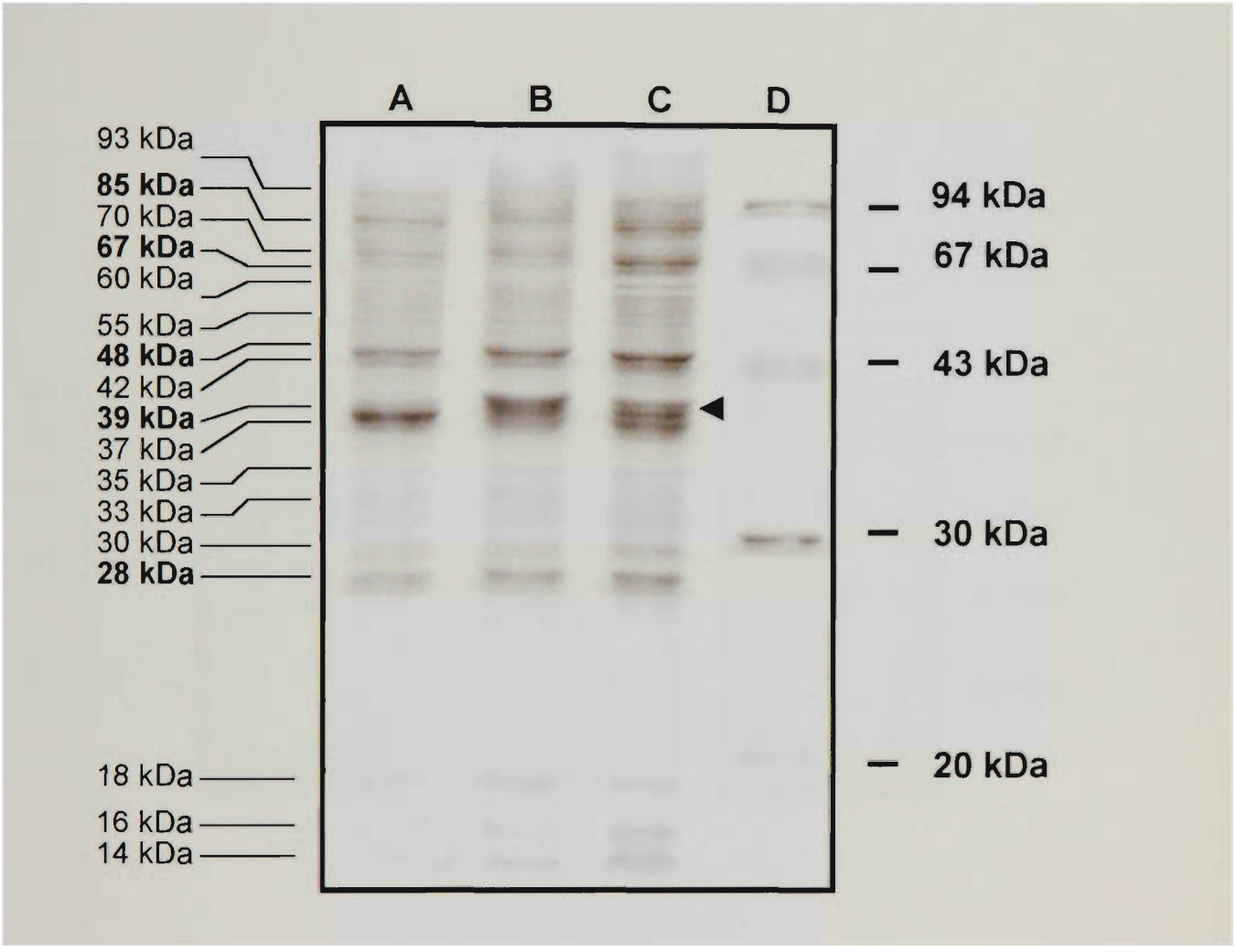


Figure 3.1 Polypeptide fingerprint analysis of *A. pleuropneumoniae* type strains on a SDS-PAGE gel. Whole cell bacteria, after resuspension in 1x Laemmli reducing mix and boiling, were run on a 12% SDS-polyacrylamide gel and stained with Coomassie blue. Lane A, *A. pleuropneumoniae* type strain 1 (HS54); Lane B, *A. pleuropneumoniae* type strain 7 (WF83); Lane C, *A. pleuropneumoniae* type strain 12 (HS143); Lane D, molecular weight standards (in kDa). Arrowhead indicates the position of the 39 kDa outer membrane protein (OMP). Bold fonts (left of figure) indicate the major bands.

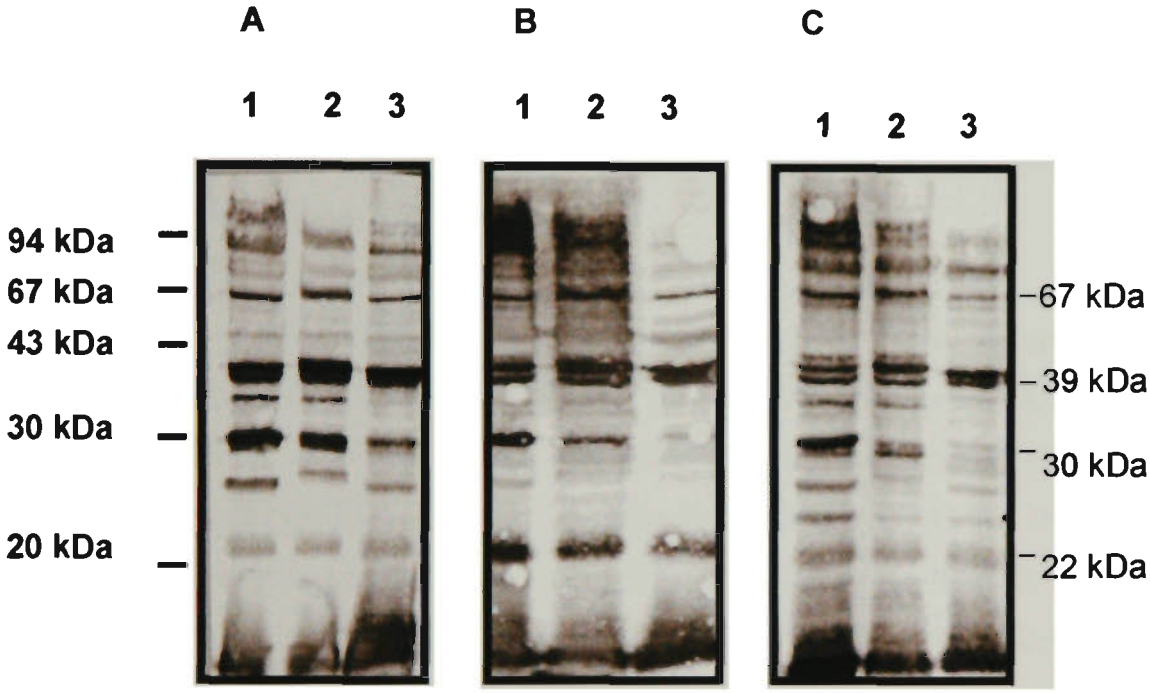


Figure 3.2 Immunoblots (A, B and C) depicting the immunoreactive protein bands of *A. pleuropneumoniae* serovar 12 (type strain HS143), lane 1; serovar 7 (type strain WF83), lane 2; and serovar 1 (type strain HS54), lane 3. Blot A was reacted against sera from pigs vaccinated with *A. pleuropneumoniae* serovar 1(HS54). Blot B was reacted against sera from pigs vaccinated with *A. pleuropneumoniae* serovar 7(WF83). Blot C was reacted against sera from pigs vaccinated with *A. pleuropneumoniae* serovar 12 (HS143). The immunoreactive common bands are labeled on the right. Control antibody was not tested. Sera were kindly provided by Dr. Chin, Dr. Eamens and Dr. Giles at EMAI).

3.3.3 Extraction of OMPs from type strains representing serovars 1, 7 and 12

To identify whether the four immunoreactive antigens (22, 30, 39 and 67 kDa in Fig. 3.2) associated with each serovar were OMPs, total membrane vesicles (TMV) were prepared from bacteria representing each of the 3 type strains. The TMV were then subjected to SLS treatment to solubilize inner membrane proteins while outer membrane vesicles were recovered intact by ultracentrifugation. Fig 3.3 shows the presence of a 22, 30, 39 and 67 kDa OMPs for Sv1. The profiles of OMPs were similar also for Sv7 and 12 (Gardiner, 1996).

3.3.4 Immunoreactivity of common OMPs of *A. pleuropneumoniae* serovars 1, 7 and 12

The immunoreactivity of OMPs extracted from *A. pleuropneumoniae* serovars 1 (HS54), serovar 7 (WF83), and serovar 12 (HS143) were analyzed by immunoblotting. OMP fractions obtained from each serovars was denatured, electrophoresed by SDS-PAGE and transferred to nitrocellulose membrane. Fig 3.4 shows the immunoreactivity of the OMPs of the 3 type strains following reaction with pooled serum from pigs that had been hyper-vaccinated against HS54 (Sv1, panel A) or WF83 (Sv 7, panel B) or HS143 (Sv 12, panel C). The most prominent immunoreactive bands had apparent molecular sizes of 30 kDa, 32 kDa, 39 kDa, 48 kDa and 67 kDa. These bands were consistently found to be immunoreactive for all 3 type strain OMPs against sera generated against each of the 3 type strains.

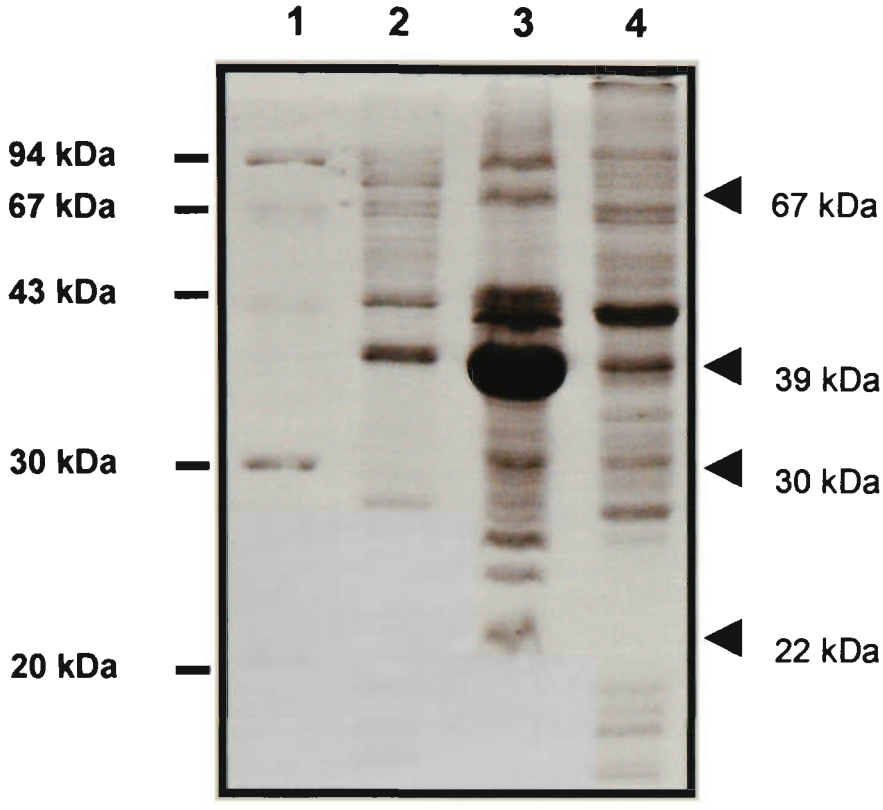


Figure 3.3 SDS-PAGE profiles of *A. pleuropneumoniae* bacteria and membrane fractions in 12% acrylamide stained with Coomassie blue. Lane 1, molecular standard markers (in kDa); Lane 2, HS54 whole cell; Lane 3, outer-membrane vesicles resolved by sucrose density centrifugation and Lane 4, inner-membrane proteins extracted by sarkosyl extraction. Arrowheads indicate the position of four major OMPs.

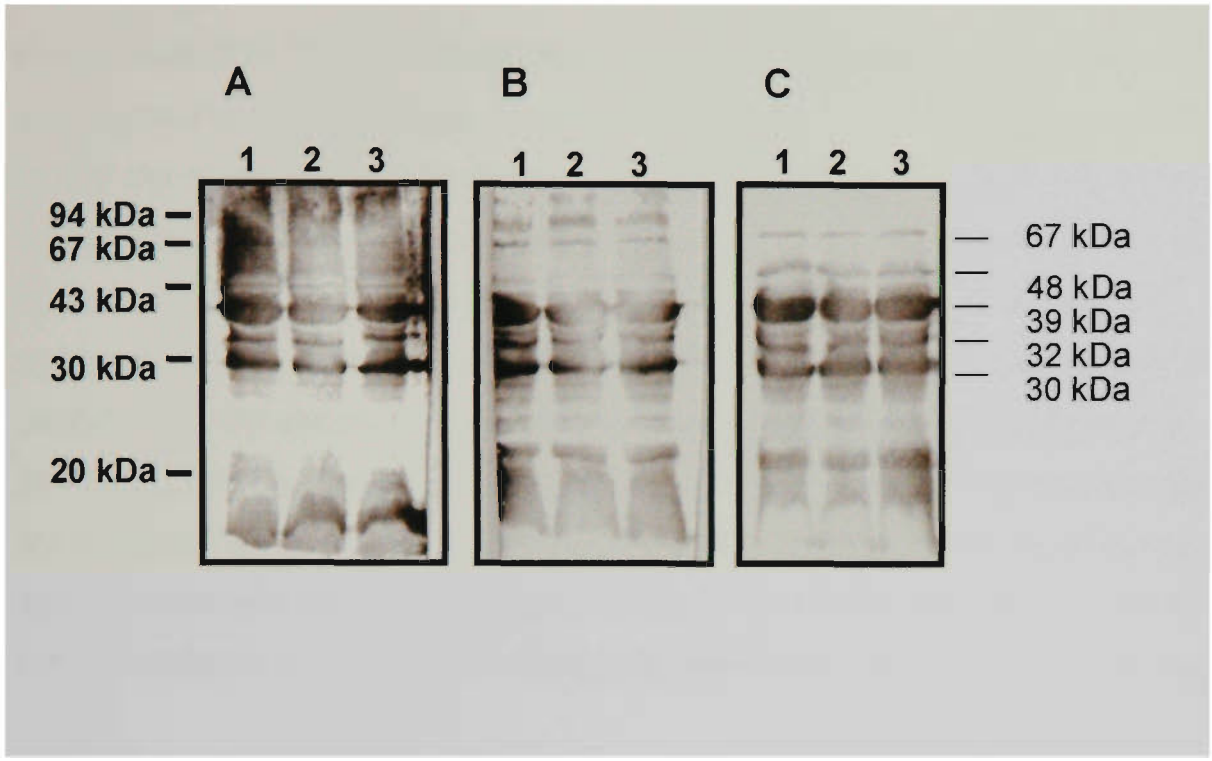


Figure 3.4 Immunoblot depicting the immunoreactivity of OMPs extracted from serovars 1 (type strain HS54), 7 (type strain WF83) and 12 (type strain HS143) (depicted in lanes 1, 2 and 3 respectively) against sera from pigs vaccinated with different *A. pleuropneumoniae* serovars. 10 μg OMP preparation was loaded onto a 12% SDS-PAGE gel and after transfer onto nitrocellulose, reacted against sera from pigs that have been vaccinated with *A. pleuropneumoniae* serovar 1, 7 or 12. Blot A was reacted against sera from animals vaccinated with *A. pleuropneumoniae* serovar 1 (HS54). Blot B was reacted against sera from animals vaccinated with *A. pleuropneumoniae* serovar 7 (WF83). Blot C was reacted against sera from animals vaccinated with *A. pleuropneumoniae* serovar 12 (HS143). The apparent molecular sizes of the common immunoreactive OMPs are presented on the right.

3.3.5 Purification of the 39 kDa OMP subunit antigen by Preparative SDS-PAGE

Preparative SDS-PAGE method was used to resolve various antigens of *A. pleuropneumoniae* whole cell, including the 39 kDa OMP. Cell pellet corresponding to 100 mg in wet weight was denatured by resuspension in Laemmli's reducing mix and boiling, after which the suspension was run on a preparative SDS-PAGE column. A total of 50 fractions were collected, and every fourth fraction was analyzed by a 10% acrylamide (v/v) SDS-PAGE gel stained using silver nitrate method. Fig. 3.5 shows the presence of various sized subunit antigens of *A. pleuropneumoniae* ranging from less than 14 kDa to 40 kDa in size. Included in those fractions were bands that resembled 33-40 kDa proteins, as analyzed against molecular weight standards. Fractions that contained those antigens were further analyzed in immunoblot assays to identify the immunodominant antigens.

Fractions containing subunit antigens resolved by preparative SDS-PAGE that were observed to have molecular weights between 33 kDa and 40 kDa were selected, and re-run on a SDS-PAGE gel. These were then transferred onto nitrocellulose and immunoblotted. After blocking, immunoreactive subunit antigens were visualized by first reacting the blots with *A. pleuropneumoniae* serovar 1 vaccinated pig sera, followed by horseradish-peroxidase conjugated goat anti-swine IgG and diaminobenzidine as HRP substrate. After the addition of substrate, a band corresponding to 39 kDa OMP was observed in fractions 46–50 (fig. 3.6). No other immunoreactive bands were visible. Fractions corresponding to the 39 kDa OMP (46-50) were pooled together and stored at -20°C. To further confirm the location of the 39 kDa OMP in the fractions obtained, fractions were profiled using ELISA to identify immunoreactive fractions that appeared to carry the 39 kDa OMP. Earlier results obtained from immunoblots had shown immunoreactive bands corresponding to 39 kDa OMP to be present in fractions 46 to 50 (fig. 3.6).

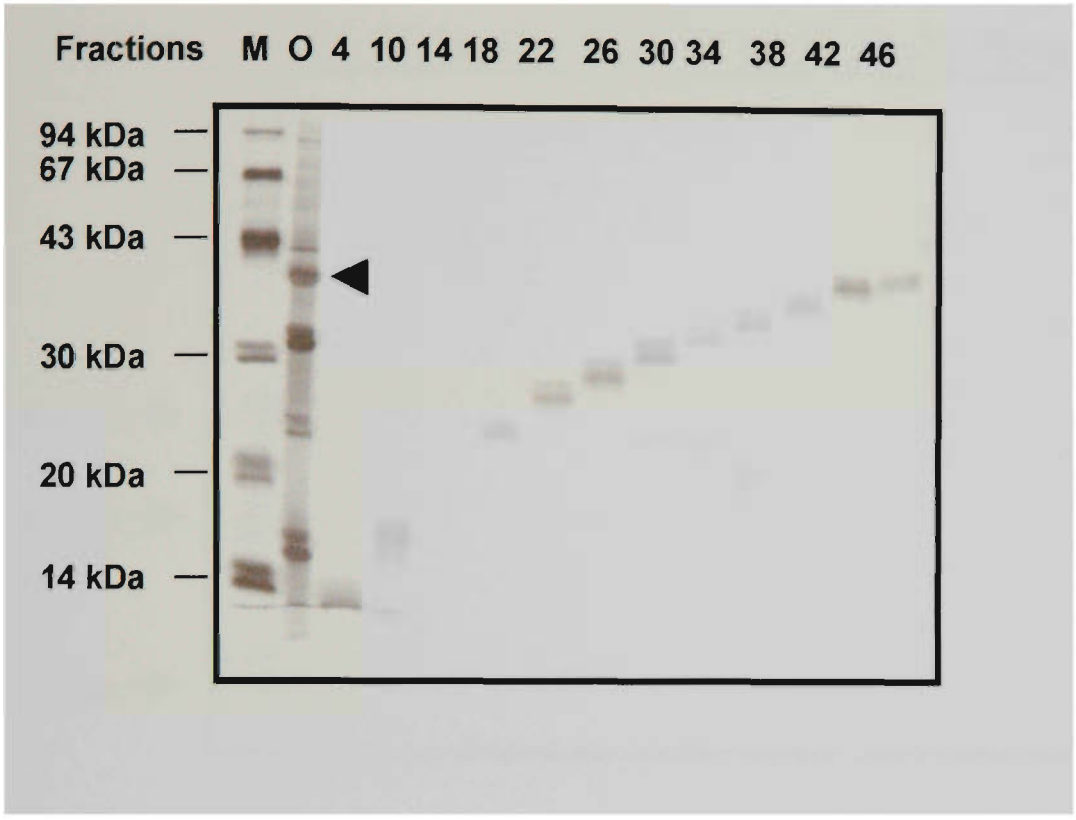


Figure 3.5 Silver stained SDS-PAGE gel depicting subunit antigens of whole cell *A. pleuropneumoniae* fractionated by preparative SDS-PAGE. Fractions collected after the preparative SDS-PAGE run were analyzed on a 10% SDS-PAGE gel by first adding 4x Laemmli reducing mix, boiling the sample, and then loading 30 μl of every fourth fraction. The protein bands were visualized by silver staining method. Arrows indicate the position of the 39 kDa OMP. Fraction numbers are presented above each lane. Lane 1 contains molecular weight standards. Fractions containing the 39 kDa antigen are observed in fractions 46 and 50. "M" represents Standard Markers; "O" represents outer membrane proteins of *A. pleuropneumoniae*.

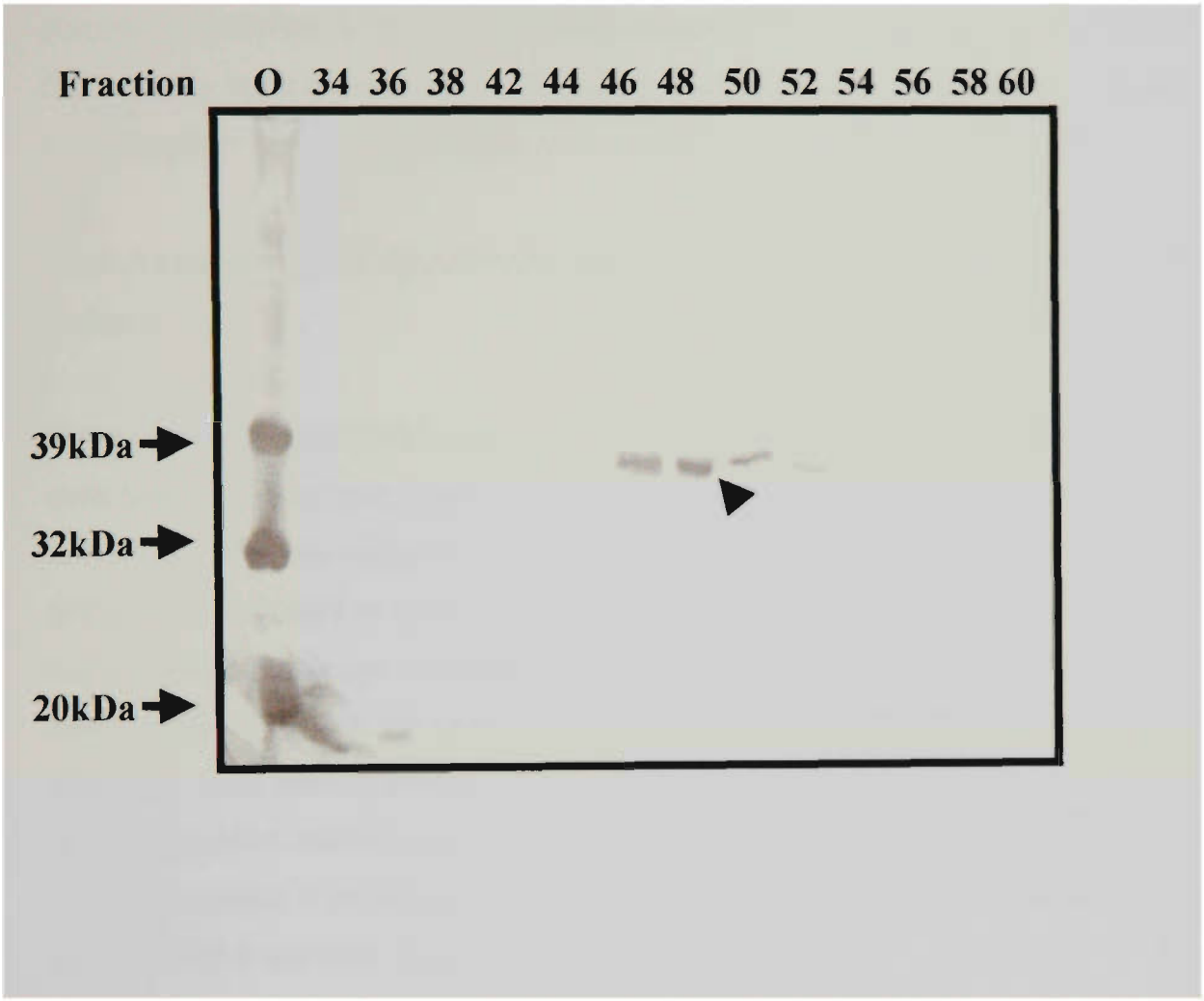


Figure 3.6 Immunoblot depicting the 39 kDa OMPs of *A. pleuropneumoniae* fractionated by preparative SDS-PAGE. Every second fraction was selected and re-run on a 10% acrylamide SDS-PAGE gel. The proteins were then transferred onto nitrocellulose and reacted with sera from pigs vaccinated with *A. pleuropneumoniae* serovar 1 (HS54). Lane numbers represent the corresponding fraction numbers respectively. "O" represents purified OMP sample. The arrowheads represent the corresponding 39 kDa immunodominant OMPs. The presence of the 39 kDa protein is observed in fractions 46-50 as indicated by single bands in those lanes. A separate control antibody was not required for the purposes of this experiment.

Proteins in fractions observed to contain the 39 kDa OMP were coated onto ELISA plates. These were then reacted with sera from pigs hypervaccinated with *A. pleuropneumoniae* serovar 1. Results showed strong reactivity (indicated by ELISA OD >0.5 as compared to low ELISA ODs of other reactive fractions) in fractions 46-50 corresponding to the location of the immunodominant 39 kDa OMP (fig. 3.7).

3.3.6 Assessment of the 39 kDa OMP of A. pleuropneumoniae as an ELISA antigen

The purified 39 kDa OMP was assessed as an ELISA reagent by reacting it against sera from pigs that were experimentally challenged with *A. pleuropneumoniae* (fig. 3.8). Animals were either challenged with *A. pleuropneumoniae* serovar 1, 7 or 12. Sera were collected at day 0 (pre challenge) and weekly thereafter. Each pen consisted of challenge animals as well as control contact animals that had not been challenged with the pathogens but were in constant contact with the infected pigs. Fig. 3.8A shows the ELISA reactivity of sera from serovar 1 (HS54) challenged and contact pigs against the 39 kDa antigen. In this case, none of the contact showed a serological response while the challenged pigs displayed a strong ELISA reaction against the 39 kDa OMP.

The immunoreactivity against the 39 kDa OMP antigen by sera from animals challenged with serovar 7 (Fig. 3.8B) and contact animals housed in the same pen yielded a different result. While immunoreactivity against the 39 kDa OMP antigen was strong in both groups of animals, there was no significant difference in their immunoreactivity against the antigen. The immunoreactivity increased from day 0 to day 4 of challenge (and contact) and once it peaked at day 14, it leveled off for the rest of the trial period (until day 42).

Fig 3.8C shows the immunoreactivity of the 39 kDa OMP antigen against sera from animals challenged with serovar 12 (HS143) and contact animals housed in the same pen with the challenged animals. The ELISA results showed that the 39 kDa

antigen was immunoreactive against both groups. There was no significant difference in the immunoreactivity between the challenge group and contact group. Immunoreactivity increased from day 0 to day 14 after challenge and remained constant until day 42 after challenge.

The immunoreactivity of the purified 39 kDa OMP was further assessed by reacting the antigen against sera from 12 randomly selected healthy piglets that had been brought into a commercial piggery with endemic pleuropneumonia. Serum was collected at days 28, 60, 92 and 128 after introduction into the piggery. The immunoreactivity shown by the 39 kDa OMP antigen was then compared against the immunoreactivity shown against *A. pleuropneumoniae* serovar 1 whole cell used as an ELISA antigen by the same sera.

Fig. 3.9A depicts the immunoreactivity of the 39 kDa OMP antigen against sera from infected pigs at days 28, 60, 92, and 128 as measured by ELISA. An increase in immunoreactivity, as indicated by higher ELISA reactivity, was observed with increasing time and maximum ELISA reactivity was observed for most pigs at days 92 and 128. Fig. 3.9B depicts the immunoreactivity of the *A. pleuropneumoniae* whole cell antigen against sera from infected pigs at days 28, 60, 92 and 128 as measured by ELISA. This was carried out in order to validate the efficiency of the 39 kDa subunit antigen as a possible diagnostic reagent. The 39 kDa antigen was observed to be immunoreactive during the course of the infection. Results showed that while the immunoreactivity of the 39 kDa OMP antigen was low compared to the whole cell antigen during days 28 and day 60, the immunoreactivity shown by the 39 kDa OMP was comparable to *A. pleuropneumoniae* whole cell when used as an ELISA antigen at days 92 and 128 (fig. 3.9B).

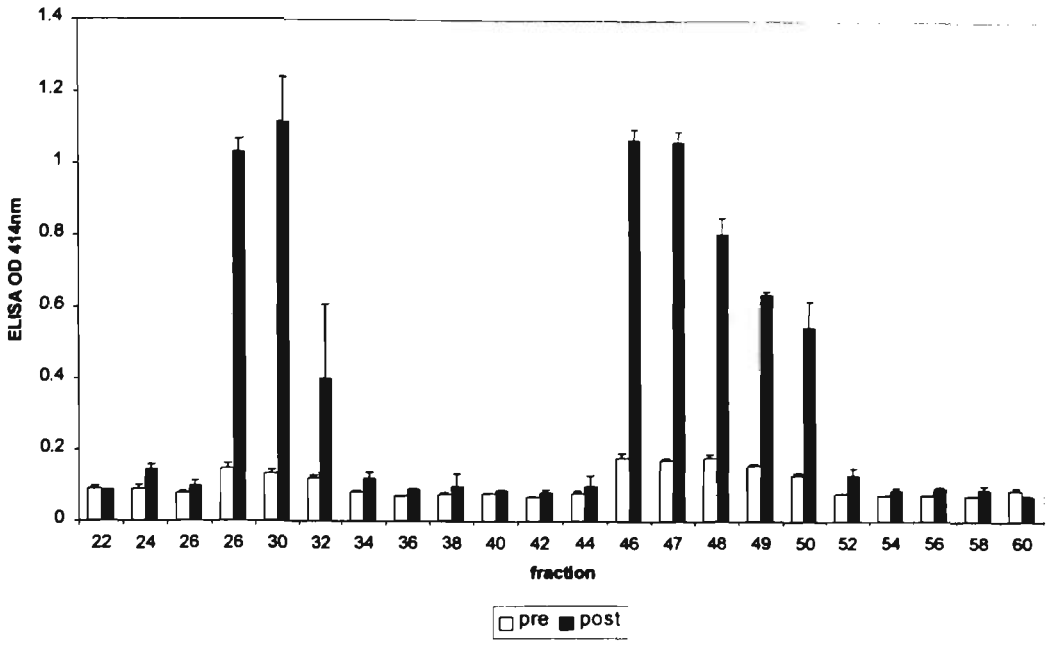
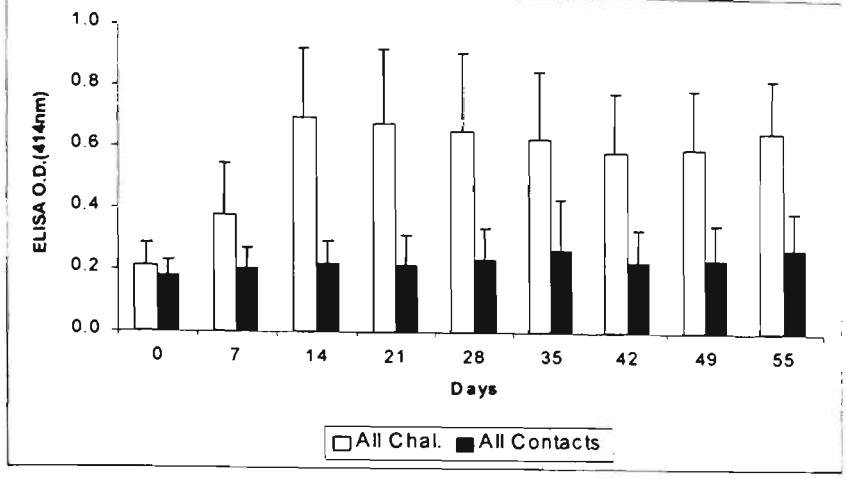


Figure 3.7 ELISA profiling of *A. pleuropneumoniae* serovar 1 (HS54). Fractions obtained from preparative SDS-PAGE were taken and diluted to 2ug/ml. After coupling onto ELISA plates, samples were first reacted against sera from pigs challenged with *A. pleuropneumoniae* serovar 1 (HS54), and then against goat anti-swine IgG labeled with horseradish peroxidase. Serum from pigs prior to *A. pleuropneumoniae* challenge was taken as negative control and is indicated by the white bars and labeled as "pre". Dark bars depict post challenge pig sera, labeled as "post". Error bars represent standard deviation of each set of data from the mean value for the set.



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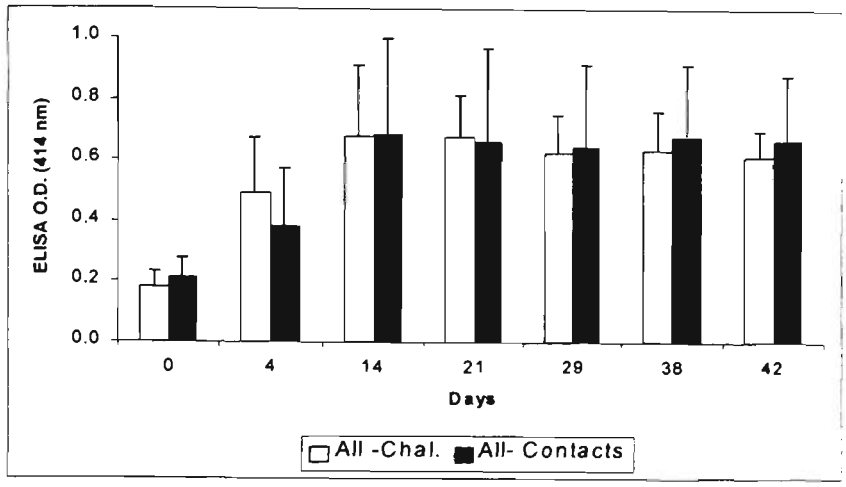
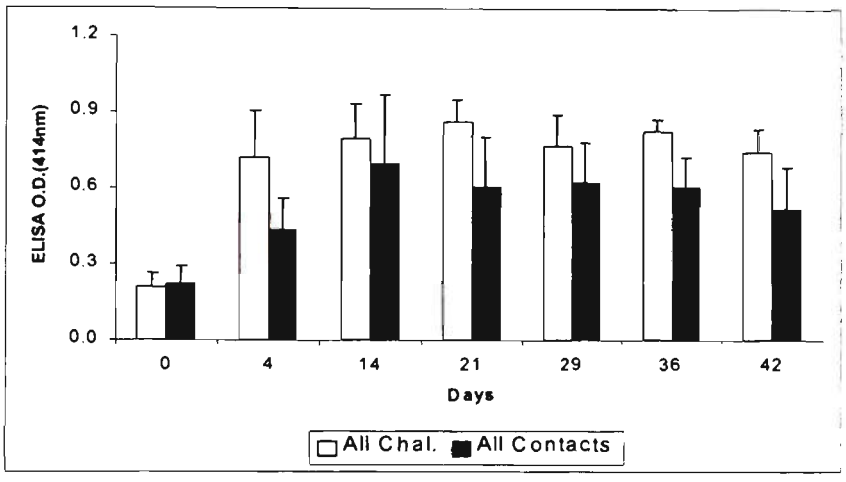


B



C

Fig 3.8 Assessment of 39 kDa OMP as an ELISA antigen. Healthy pigs were either challenged with *A. pleuropneumoniae* serovars 1 (HS54), 7 (WF83) and 12 (HS143) or used as contact controls in the same pen. (A) Represents immunoreactivity against 39 kDa OMP by pig sera from animals challenged with *A. pleuropneumoniae* serovar 1 at different time points, day 0 being pre-challenge sera, as assessed by ELISA. (B) Represents immunoreactivity against 39 kDa OMP by pig sera from animals challenged with *A. pleuropneumoniae* serovar 7. Similarly, (C) represents immunoreactivity against 39 kDa OMP by pig sera from animals challenged with *A. pleuropneumoniae* serovar 12. Error bars represent standard deviation of each set of data from the mean value for the set. [I would like to acknowledge the contributions of Dr.Graeme Eamens (in providing the sera), Dr.James Chin (providing sera), and Bernadette Turner (hands on assistance), in this ELISA assessment experiment].

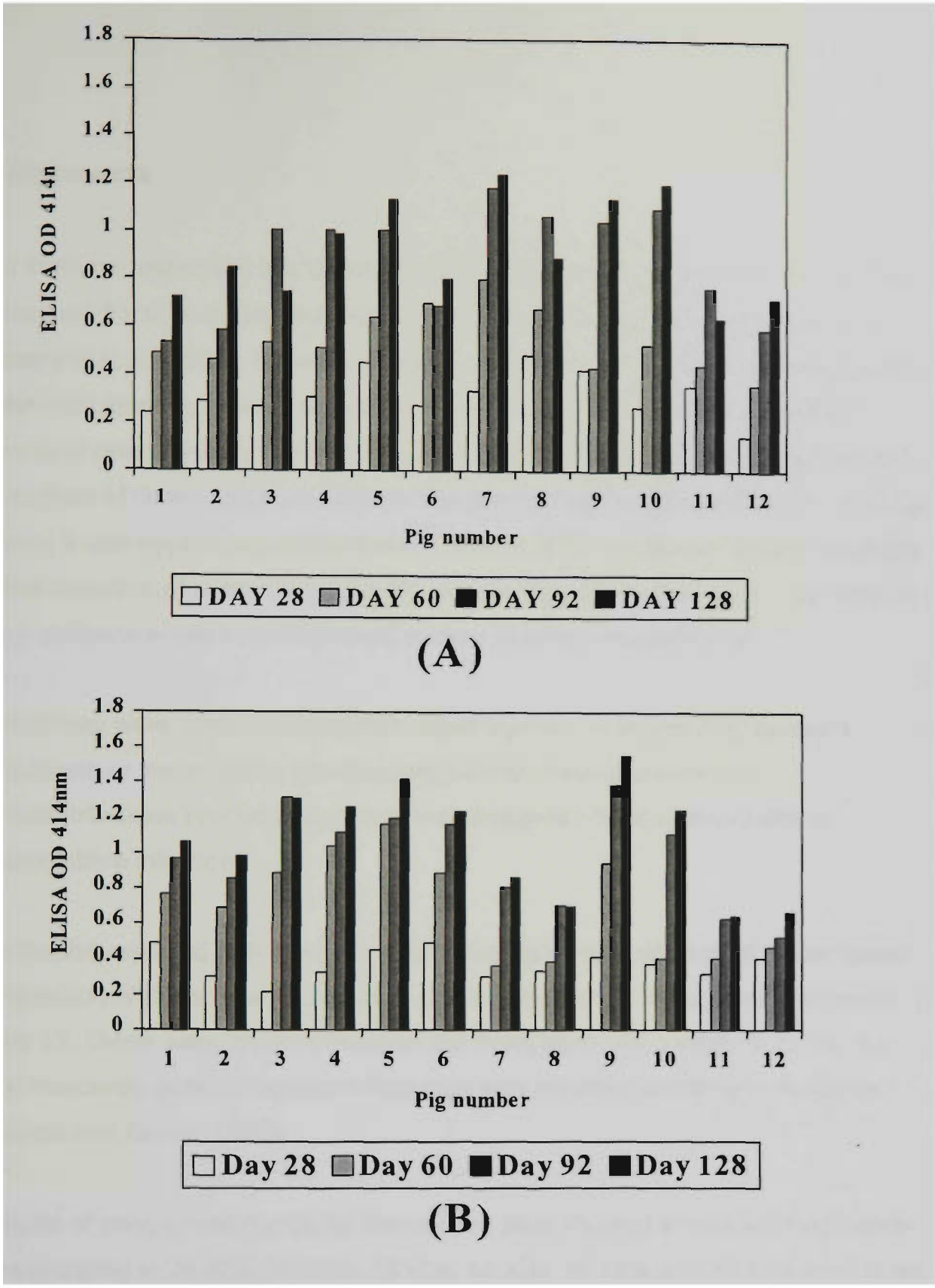


Figure 3.9 Assessment of 39 kDa OMP as an ELISA antigen. Pig sera were obtained from a commercial piggery with endemic pleuropneumonia and reacted with an antigen panel consisting of **(A)** purified 39 kDa OMP antigen and **(B)** *A. pleuropneumoniae* whole cells. The pig sera were obtained from pigs that had initially been introduced into the piggery as healthy pigs and had been housed there for 28 days, 60 days, 92 days, and 125 days respectively. Immunoreactivity was detected by standard ELISA using HRP conjugated goat anti-swine IgG. The immunoreactivity against *A. pleuropneumoniae* whole cell was monitored as a positive control. (I would like to thank Dr. James Chin for providing the sera, and to Bernadette Turner for assisting in some of the ELISA procedures).

3.4 Discussion

Host immune responses are directed mainly against surface antigens of invading pathogenic bacteria during the primary encounter between host and pathogen (Costerton *et al.*, 1981). In Gram-negative bacteria, front-line antigens recognized by the host immune system are usually those arrayed in the outer membrane, commonly referred to as OMPs (Chin & Dai, 1989). Since their very presence on the surface of Gram-negative bacteria renders them prime targets for the immune system, it was hypothesized that these OMPs may be useful serological reagents for the detection of antibody responses against App in infected pigs. The utility of these antigens would be determined by the following requirements:

1. that they were common antigenic determinants to different App serovars
2. that one or more OMPs are immunogenic and immunodominant
3. that antibodies produced against these antigens are associated with a productive infection

The studies reported here were directed towards an examination of the antigenic composition of three type strains of *A. pleuropneumoniae* representing serovars 1, 7 and 12. These were chosen because the three serovars constitute by far, the most frequently isolated serovars from pigs with pleuropneumonia in Australia (Blackall and Eaves, 1986).

Analysis of polypeptide bands by Coomassie Blue showed strong staining bands corresponding to 28 kDa, 30 kDa, 39 kDa, 48 kDa, 67 kDa and 85 kDa in all three serovars. Other reports have shown that the 42 kDa, 39 kDa, 29 kDa and 16 kDa bands were darkly stained in App serovars 1 to 9 (Rapp *et al.*, 1986). Of these, the 28kDa, the 39 kDa, and the 48 kDa were confirmed to be OMPs when purified by the Sarkosyl protocol (Rapp *et al.*, 1986; Thwaites *et al.*, 1991).
Having confirmed the presence of common dominant antigens in all three serovars, it was important to demonstrate that some of these antigens were indeed outer

membrane proteins. This was accomplished using the procedure of Chin and Turner (1990). The method used for the extraction involved solubilization of the inner membrane vesicles in a TMV complex made up of OMVs and IMVs. This allowed the extraction of the OMVs from the IMV complex. Further high-speed centrifugation separated the OMVs from IMPs. The extraction yielded a number of OMP bands on SDS-PAGE gel, including a prominent darkly stained 39 kDa OMP band (fig. 3.3). Other bands included an 85 kDa, 75 kDa, a 48 kDa, a 42 kDa, a 32 kDa and a 28 kDa band. The presence of the 39 kDa bands in the outer membrane fractions of all three serovars confirmed the finding by Rapp and colleagues (1986) that the 39 kDa OMP of *A. pleuropneumoniae* is conserved across all serovars.

When the OMP preparations were reacted against sera from pigs challenged with each of the three serovars, several bands showed immunoreactivity, including the 39 kDa OMP band, regardless of the serovar from which the OMPs were extracted. This was consistent with the results of Rapp and colleagues (1986) showing the immunodominance of the 39 kDa OMP. This finding is also consistent with previous studies where it was observed that OMP preparations from three *A. pleuropneumoniae* serovars (1, 5 and 7), when reacted with sera from pigs challenged with serotype 5, showed the presence of a prominent band resembling the 39 kDa OMP (Rapp & Ross, 1986).

The presence of the 39 kDa antigen across the serovars and its strong immunoreactive nature made it an ideal ELISA candidate. The 39 kDa OMP from the three serovars was purified using a method known as preparative SDS-PAGE. The use of preparative SDS-PAGE is recent. Chin & Djordjevic (BIO-RAD bulletin 2036) have been able to identify and purify subunit antigens of *Mycoplasma hyopneumoniae* and *Erysipelothrix rhusiopathiae* using this procedure. Scarman *et al.* (1997) have shown the efficacy of using this procedure in the comparison of protein and antigenic profiles of three porcine pathogens, *Mycoplasma hyopneumoniae*, *Mycoplasma hyorhinis* and *Mycoplasma flocculare*. This group developed and evaluated a preparative SDS-PAGE profiling technique that combined the advantages of the electrophoretic separation of proteins

with the convenience and speed of ELISA to quantify immunoreactivity of certain antigens.

Using this method, *A. pleuropneumoniae* cell pellet was fractionated into various molecular sizes and the fraction containing the 39 kDa soluble OMP antigen identified based on the molecular size of the protein on a SDS-PAGE gel. The identity of the 39 kDa band was further confirmed by immunoblotting where the different fractions were reacted against sera from pigs vaccinated with *A. pleuropneumoniae* serovar 1(HS54). The 39 kDa band was visible in the immunoblot and was located in fractions 46-50.

The 39 kDa OMP antigen was reacted against sera from pigs that had been challenged with three different *A. pleuropneumoniae* serovars (serovars 1, 7 and 12). The aim was to observe and assess the immunoreactivity of the sera against the 39 kDa antigen. Animals challenged with serovar 1, 7 and 12 showed strong immunoreactivity against the 39 kDa OMP antigen within a short period after challenge. The immunoreactivity against the 39 kDa OMP was significantly better ($P<0.05$, two tailed T-test) by sera from animals challenged with *A. pleuropneumoniae* serovar 1 (HS54) than by sera from contact animals after the first week of challenge, continuing until 6 weeks after challenge. In this case, the immunoreactivity against the 39 kDa OMP antigen was lower ($OD < 0.5$) for contact animals at all time points.

The immunoreactivity, as quantified by ELISA, was not significantly different ($P>0.1$, two tailed T-test) between challenge and contact group for animals challenged with *A. pleuropneumoniae* serovar 7 and 12 respectively. One possible reason for this could be that the animals challenged with serovar 7 and 12 were transferred back to their herd housing unchallenged pigs immediately after challenge, while they were still unconscious. Animals challenged with serovar 1 were isolated for 24 hours after challenge before returning them to their herd housing unchallenged pigs. During this time, the innate immune response would have neutralized residual pathogen, lowering the number of pathogen transmitted to "healthy" animals. This was not true for those

pigs challenged with serovars 7 and 12, since they were more likely to transmit the higher number of residual pathogen directly to the "healthy" pigs in the herd.

The ELISA assay involving the 39 kDa OMP was further used to assess the antibody reactivity of sera from feeder pigs whose growth was being monitored over time following introduction into a piggery with endemic pleuropneumonia. Results showed that the 39 kDa OMP is recognized by antibodies present in the pig sera from day 28. This is an important result because it shows that the 39 kDa OMP can be used to detect the presence of antibodies against *A. pleuropneumoniae* early in the course of an infection. Furthermore, the immunoreactivity of the 39 kDa OMP antigen was comparable to that of *A. pleuropneumoniae* whole cell used as a control positive antigen in the experiments.

The results obtained using the 39 kDa OMP antigen as a possible diagnostic reagent have been promising. It has been shown from this research project that the 39 kDa antigen can detect the presence of *A. pleuropneumoniae* in challenged as well as infected pigs. Although membrane proteins from other gram-negative bacteria have been used as ELISA antigens (Chin and Turner, 1990; Chin and Dai, 1989), same is not true regarding membrane proteins of *A. pleuropneumoniae*. The use of the *A. pleuropneumoniae* 39 kDa OMP as an ELISA reagent has not been reported to date. To our knowledge, this is the first time the 39 kDa OMP of *A. pleuropneumoniae* has been trialed in such an assay. The results obtained from this work have only shown that the 39 kDa antigen is useful in detecting *A. pleuropneumoniae* during the course of an infection. The questions of sensitivity as well as species-specificity have to be addressed in future trials involving this antigen. The issues of cross reactivity as well as non-reactivity of sera from pigs infected with other members of HAP family of bacteria against the 39 kDa OMP antigen have to be investigated as well. It can be stated without doubt that further trials are necessary to fully establish the significance of using the 39 kDa OMP as an ELISA antigen.

Chapter 4: Characterization, purification and serological evaluation of a recombinant toxin- ApxIV

4.1 Introduction

A. pleuropneumoniae strains are known to produce at least one or a combination of three extracellular toxins - ApxI, ApxII and ApxIII (Frey, 1994; Frey *et al.*, 1993; Frey *et al.*, 1994). The combination of secreted toxin or hemolysin is characteristic for a given serotype (Beck *et al.*, 1994) and it has been proposed that the virulence of each strain is highly dependent upon the combination of exotoxins secreted (Frey *et al.*, 1993).

ApxI is secreted by serotypes 1, 5a, 5b, 9, 10 and 11 (Beck *et al.*, 1994) while ApxII is produced by all serotypes except serotype 10 (Kamp *et al.*, 1991). ApxIII is secreted by serotypes 2, 3, 4, 6 and 8 (Kamp *et al.*, 1991). ApxI and ApxII are hemolytic. ApxIII is cytotoxic to pig neutrophils. All three exotoxins belong to the family of RTX toxins, which includes the hemolysin toxin of *E. coli*, leukotoxins of *Pasteurella* and *Actinobacillus*, and the adenylate cyclase-hemolysin of *Bordetella pertussis*. The RTX toxins contain a hydrophobic domain in the N-terminal, a calcium-binding domain in the C- terminal and a lysine rich region involved in the acylation of the inactive pro-hemolysin (Fig. 1.1)(Stanley *et al.*, 1998).
Thompson *et al* (1994) reported that *Neisseria meningitidis* also produces two iron-regulated proteins (FrpA and FrpC) related to the RTX family of exoproteins. While investigating the expression and phylogenetic relationships of a novel *lacZ* gene in *A. pleuropneumoniae*, Anderson and McGuinnes (1997) were able to identify sequences similar to the *frpA* and *frpC* genes of *Neisseria meningitidis* downstream of the *lacZ* gene in *A. pleuropneumoniae*. The possibility that this homology might encode a new Apx protein was further explored by Schaller *et al.*, (1999) who were successful in cloning and expressing the new Apx gene – *apxIV* in *E.coli*.

According to Schaller *et al.* (1999), the *apxIV* gene was detectable by DNA hybridization in all *A. pleuropneumoniae* strains. However, various strains may produce different polymorphic forms of the toxin. For example, sequence data of the *apxIV* gene from the 2 type strains Shope 4074 (serovar 1) and HV114 (serotype 3) show that *apxIV* gene encodes toxins with molecular sizes of 202 and 170 kDa respectively.

The role of ApxIV in pathogenesis has not been elucidated. Yet, the toxin appears to have an extremely interesting property. Unlike other Apx toxins, ApxIV is never produced during *in vitro* culture by virulent strains of *A. pleuroponeumoniae*. Its production in the course of *in vivo* infection is only documented indirectly from immunochemical data where sera from infected pigs, were found to react in immunoblots against the ApxIV antigen expressed by *E. coli*. This finding clearly needs further corroboration and would be of immense value as a diagnostic antigen for the detection of infection by *A. pleuropneumoniae* if antibody responses against ApxIV could be quantified in an ELISA test.

There are very few published studies where Apx toxins have been used in an indirect ELISA test. To develop such an ELISA, Devenish *et al.*, (1990) had to firstly purify Apx from Ap serovar 1 strain CM-5. The purified toxin (104 kDa, supposedly ApxII) was then used as an immunogen to generate rabbit polyclonal antibodies from which the toxin-specific IgG was affinity purified. The purified rabbit anti-Apx was then used as the coating antibody in ELISA plates to capture Apx protein from crude bacterial supernatants. Ma and Inzana (1990) also used a similar approach to generate a 110 kDa toxin (supposedly ApxI) from Ap serovar 5 strain J45. However, since serovars 1 and 5 are known to secrete both ApxI and II, it is not clear from these reports which of the two antigens were being captured and used in the ELISA test. The quality control procedures to confirm the identity of either ApxI or ApxII were simply not carried out. It is evident that ELISA assay reagents produced by complex procedures require extensive quality control steps to be implemented before the antigen can be utilized as a diagnostic reagent. The

establishment of a toxin-based ELISA would be very much simplified if an easier and less time consuming procedure were to be developed for the production of Apx toxin antigen.

Another factor that may interfere with the use of a toxin based ELISA assay is the possibility of cross-reactivity with toxins produced by species other than *A. pleuropneumoniae*. For instance, sera from pigs infected with *A. suis* (known to produce a toxin identical to ApxII of *A. pleuropneumoniae* , Burrows and Lo,1992) reacted with ApxI/II toxin from serotype 1 and failed to against ApxI/II prepared from serotype 5 (Kamp *et al.*, 1994). Such observations render the ELISA test less specific in its diagnostic capability. Unlike Apx I, II and III, the observation that the *apxIV* gene is present in all *A. pleuropneumoniae* serovars endorses the possibility that the ApxIV antigen is common to all strains. Consequently, the use of purified recombinant ApxIV (rApxIV) as an ELISA reagent will provide an additional supporting test to the 39 kDa OMP antigen.

The aims of this chapter are as follows:

1. To induce the production of rApxIV from plasmids constructed to express either the C-terminal or N-terminal of ApxIV (i.e. C-ApxIV and N-ApxIV).
2. To purify C-ApxIV and N-ApxIV by Preparative SDS-PAGE.
3. To evaluate purified C-ApxIV and N-ApxIV antigens as ELISA reagents for the detection of antibodies against *A. pleuropneumoniae* in infected pigs.

4.2 Materials and Methods

This section follows methods and materials already outlined in chapter 2. Materials and methods outlined here pertain only to experiments carried out in this chapter.

4.2.1 Bacterial strains and plasmids used

Table 4.1. List of bacteria used

Bacterial strain	Genotype	Source/Reference
HMS147(DE3)	*E. coli*	Dr. J. Frey (University of Berne, Switzerland)
JM109λpir*	*E.coli*	(Yanisch-Peron *et al.*, 1985)

**E.coli* JM109λpir was used as a control *E.coli* host carrying the His-tag plasmid pQE9.

Table 4.2. List of plasmids used

Plasmid	Phenotype	Source/Reference
pJFFapxIVA1NaHis1	Ap^r/N-apxIV	Dr. J. Frey (University of Berne, Switzerland)
pJFFapxIVA3CHis1	Ap^r/C-apxIV	Dr. J. Frey (University of Berne, Switzerland)
pQE9**	Ap^r/His-tag®	Novagen

** Plasmid pQE9 was used as a His-tag control plasmid since the original expression plasmid used by Schaller and colleagues, pET14b was not available. The use of pQE9 was based on the similarity between the two plasmids in that both were capable of expressing His-tagged protein under IPTG induction, and both confer ampicillin resistance.

4.2.2 Induction of expression of recombinant protein antigens

E. coli colonies expressing the recombinant toxins (Schaller et al., 1999) were inoculated into10 ml overnight LB broth supplemented with 100 μg/ml ampicillin. A 5 ml aliquot of the overnight culture was used to inoculate 50 ml starter cultures in 250 ml Erlenmyer flasks. Growth of bacteria was monitored until start of log phase (absorbance OD of 0.5 at 650nm). At this point, the bacteria were induced with 0.5

mM or 2.0 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and grown for a further 3 hours. Bacteria were then centrifuged (5000 g, 30 min, 4°C) in a Beckman high-speed centrifuge. The pellets were weighed, aliquoted and stored at -20°C.

4.2.3 Preparative SDS-PAGE fractionation

Preparative SDS-PAGE (PAGE-PREP) technology was used to purify soluble antigen of known molecular weight from whole cell preparations. The Prep Cell was obtained from BIO-RAD (Model 491). Apparatus assembly, gel preparation, and use were all performed as per manufacturer's instructions.

E.coli HMS147(DE3) cells carrying the recombinant clones were reduced in Laemmli mixture [(5% β-Mercaptoethanol (w/v); 62.5 mM Tris-Cl pH 6.8; 3% SDS; 10% glycerol (w/v)] by boiling (Laemmli, 1970), and several crystals of bromophenol blue were added to the sample. Cell preparation was loaded onto a 9% polyacrylamide gel column (Bioprep 491 cell, Bio-Rad), 9 cm in height with a 1 cm stacking gel consisting of 4% polyacrylamide. Columns were run at 40 mA constant current (150-300 V) for 11 hours and fractions (9 ml) were collected immediately after elution of the bromophenol dye. The electrophoresis and elution buffers comprised SDS-PAGE buffer (0.1% SDS, 50 mM Tris, 384 mM glycine), and the flow rate used was 1 ml min^{-1}.

Fractions obtained by PAGE-PREP were analyzed by running minigel SDS-PAGE containing a 8% separating gel and 4% stacking gel.

4.2.4 Scan profile analysis of protein bands

Bio-rad Molecular Analyst software was used in quantitative analysis of protein bands in SDS-PAGE gels. Gels were scanned using a Bio-rad densitometer, and subsequently analyzed using Molecular Analyst software for quantifying the percentage of protein in the given lane.

4.3 Results

4.3.1 Effect of IPTG on growth of host *E. coli*

Growth of the bacteria was monitored at various time points either in the presence of 2 mM IPTG or in the absence of IPTG (fig. 4.1). Results showed reduced growth rate of the host *E. coli* expressing recombinant N-apxIV in the presence of IPTG (fig.4.1C). Growth was also reduced in the presence of IPTG in the growth culture of *E. coli* expressing recombinant C-apxIV (fig. 4.1B). Growth of *E. coli* JM109 λpir carrying plasmid pQE9 was also monitored as a negative plasmid control (fig.4.1A). Growth was not repressed significantly in the presence of IPTG during growth of this bacterium.

4.3.2 Polypeptide fingerprint analysis of *E. coli* strains in the presence and absence of IPTG

In order to express the recombinant subunit antigens (N-apxIV and C-apxIV) in the host *E. coli* HMS147(DE3), IPTG was introduced into the growth culture. Two concentrations of IPTG, 0.5 mM and 2.0 mM, was used to determine whether there was a difference in induction at these concentrations. Aliquots of bacterial growth culture were taken at 0,1, 2 and 3 hours after the start of induction. The bacterial pellets were loaded onto a SDS-PAGE gel and the polypeptide fingerprints analyzed. An 80 kDa subunit antigen corresponding to N-apxIV (Schaller *et al.*, 1999) was observed as a distinct band in the presence of IPTG at all time points, except at 0 hours, the time of start of induction (fig. 4.2A). A Molecular Analyst aided protein scan profile indicated that the amount of induced protein increased with time when 2.0 mM IPTG was used (fig. 4.3). A distinct band corresponding to a 96 kDa protein was observed after host *E. coli* expressing recombinant C-apxIV (Schaller *et al.*, 1999) was induced with 0.5 mM and 2.0 mM IPTG (fig. 4.2B). The amount of induced protein increased from 0 hours to 1 hour, although there was no gradual increase from 1 hour to 3 hours (fig. 4.3).

Based on the scan profiles of IPTG-induced recombinant bacteria, it can be calculated that the amount of induced recombinant C-apxIV and N-apxIV was 15% and 17% respectively of total protein loaded onto each lane of the SDS-PAGE gel. In the course of scaling up purification of recombinant proteins by Preparative SDS-PAGE, about 5000 μg of bacterial protein (equivalent to 100 mg wet weight of induced bacteria) is normally loaded onto each preparative gel column. From this, approximately 750 and 850 μg of recombinant C-apxIV and N-apxIV protein can be recovered. For ELISA analysis, these recombinant antigens were further diluted in coating buffer to a final concentration of about 2 μg/ml.

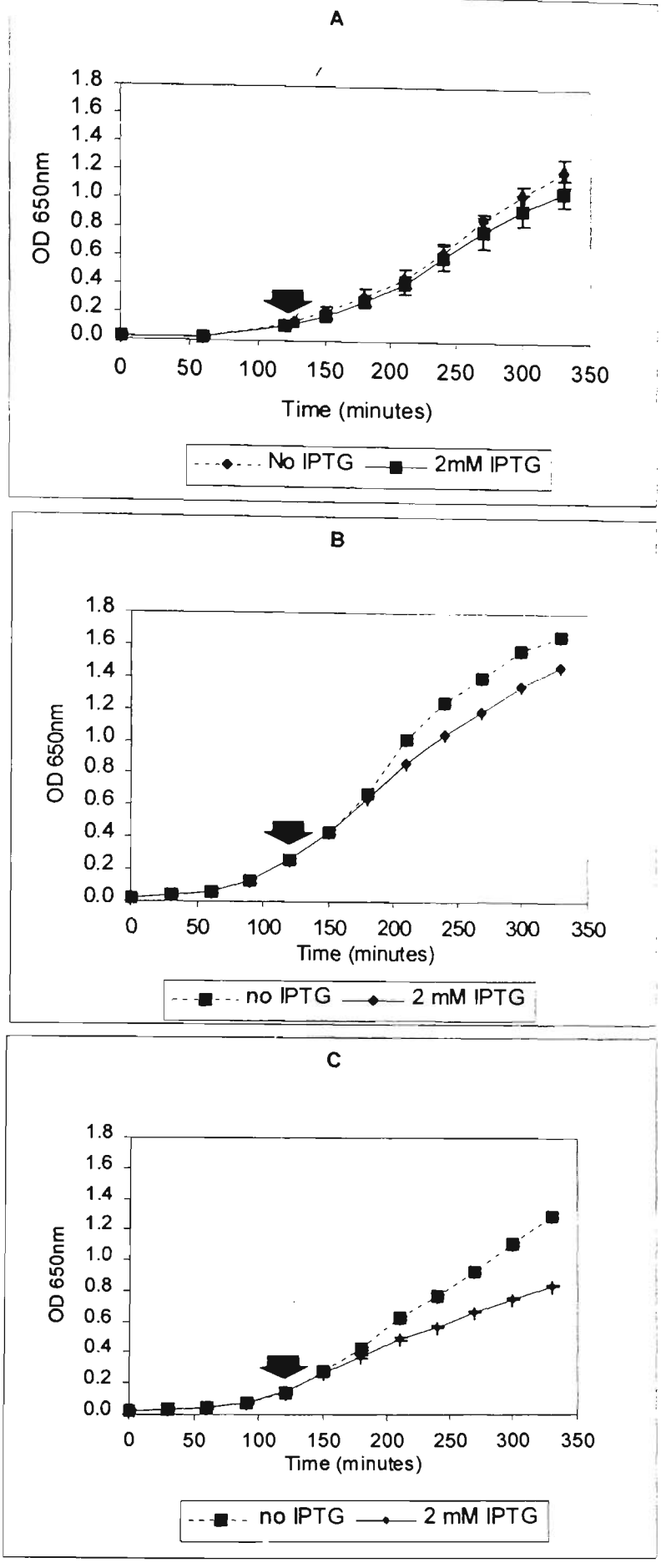


Figure 4.1 Effect of IPTG supplementation on growth of (**A**) host *E. coli* JM109 λ-pir/pQE9, (**B**) *E.coli* HMS147(DE3) expressing recombinant C-apxIV, and (**C**) *E.coli* HMS147 (DE3) expressing recombinant N-apxIV. Bacterial cells were grown in Luria broth (LB) supplemented with 50 μg/ml ampicillin and growth monitored. Samples were read on a spectrophotometer at OD 650nm and the values plotted. 2 mM IPTG was added to the growth culture at the start of log growth. Arrow indicates the time of IPTG supplementation.

Polypeptide analysis was carried out on *E. coli* JM109λnir/pQE9 grown in the absence and presence of 2 mM IPTG (figure not shown). Results showed no difference in bands corresponding to 80 kDa and 96 kDa proteins in bacteria grown under the two conditions. Furthermore, an immunoblot analysis was carried out on *E. coli* HMS147 (DE3) carrying the recombinant plasmids, the host strain *E. coli* HMS147(DE3) (no plasmid), host *E.coli* JM109λpir/pQE9 (His-tag plasmid control) and *A.pleuropneumoniae* HS54 (positive control) using serum from pigs challenged with *A. pleuropneumoniae* serovar 7. The results did not reveal the presence of bands corresponding to rApxIV proteins in *A. pleuropneumoniae* HS54, and in the other two *E. coli* strains. Immunoreactive bands corresponding to the recombinant ApxIV toxins were observed for the host *E. coli* HMS147 (DE3) expressing the recombinant toxins (fig. 4.4).

4.3.3 Purification of recombinant N-apxIV and C-apxIV subunit antigen by Preparative SDS-PAGE method

Induced host *E. coli* cells were fractionated by the preparative SDS-PAGE method as this method has been successfully used in the laboratory of Dr. J. Chin for purification of other recombinant proteins. A 6% polyacrylamide gel column was used to resolve the protein bands. The *E. coli* cells were resolved into various subunit antigens, including the 80 kDa N-ApxIV recombinant antigen in fractions 22-28 (fig. 4.5A). *E. coli* expressing the 96 kDa recombinant C-ApxIV was also subjected to fractionation on a preparative SDS-PAGE column containing a 6% polyacrylamide gel. The fractionation of this *E. coli* strain yielded various subunit antigens, including the 96 kDa recombinant antigen in fractions 34-40 (fig. 4.5B).

4.3.4 Confirmation of presence of bands corresponding to the recombinant subunit antigens by ELISA profiling

Recombinant subunit antigens N-ApxIV and C-ApxIV were purified from whole cell *E. coli* by preparative SDS-PAGE method (section 4.2.1). Fractions were chosen based on earlier results from section 4.3.3, and coupled onto ELISA plates. Serum from pigs challenged with *A. pleuropneumoniae* serovar 7 was then used to react against the recombinant antigen. Fractions that showed reactivity against the antisera, as confirmed by horseradish peroxidase labeled goat anti-swine IgG binding, were identified. Fractions 21-28, corresponding to presence of the recombinant protein N-apxIV (fig. 4.6A), showed reactivity. ELISA profile of C-apxIV is depicted in fig. 4.6B. Fractions 35-40, corresponding to presence of recombinant protein C-ApxIV showed reactivity against the antisera.

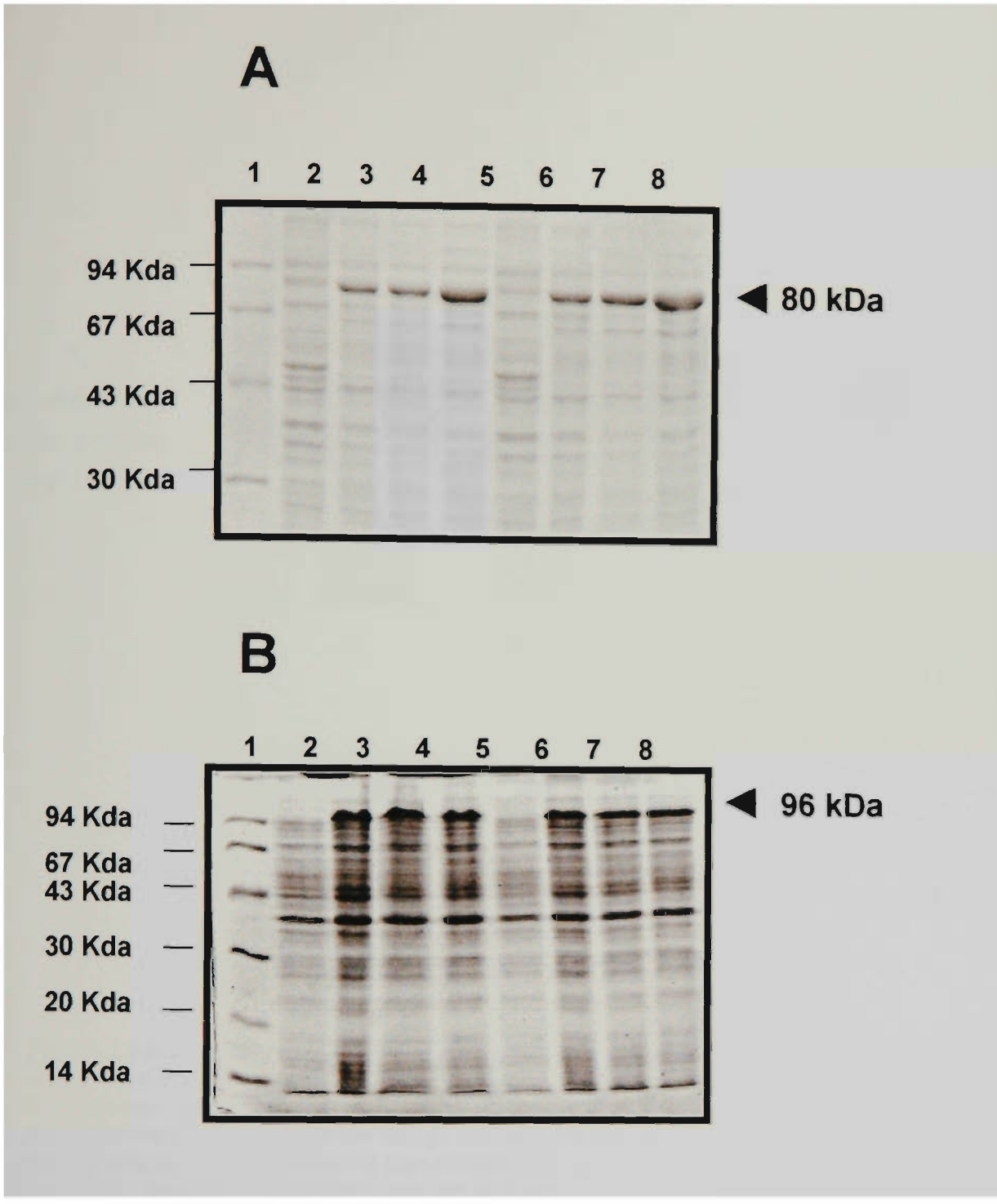


Figure 4.2 Polypeptide fingerprints after IPTG supplementation on host bacteria *E. coli* HMS147(DE3) expressing the (**A**) 80 kDa recombinant N-apxIV and (**B**) 96 kDa recombinant C-apxIV. Cells were induced with 0.5 mM and 2 mM IPTG and their growth monitored over time. At times 0, 1, 2 and 3 hours, aliquots were taken and these were pelleted and after resuspension in Laemmli reducing mix they were loaded onto an 8% SDS-polyacrylamide gel. Loading was standardized at 5 μg protein per lane. Lane 1, molecular weight standards. Lane 2-5 represent 0.5 mM IPTG induction at times 0, 1, 3 and 5 hours respectively. Lane 6-9 represent 2.0 mM IPTG induction at times 0,1, 3 and 5 hours respectively. Arrows represent the induced recombinant subunit antigens.

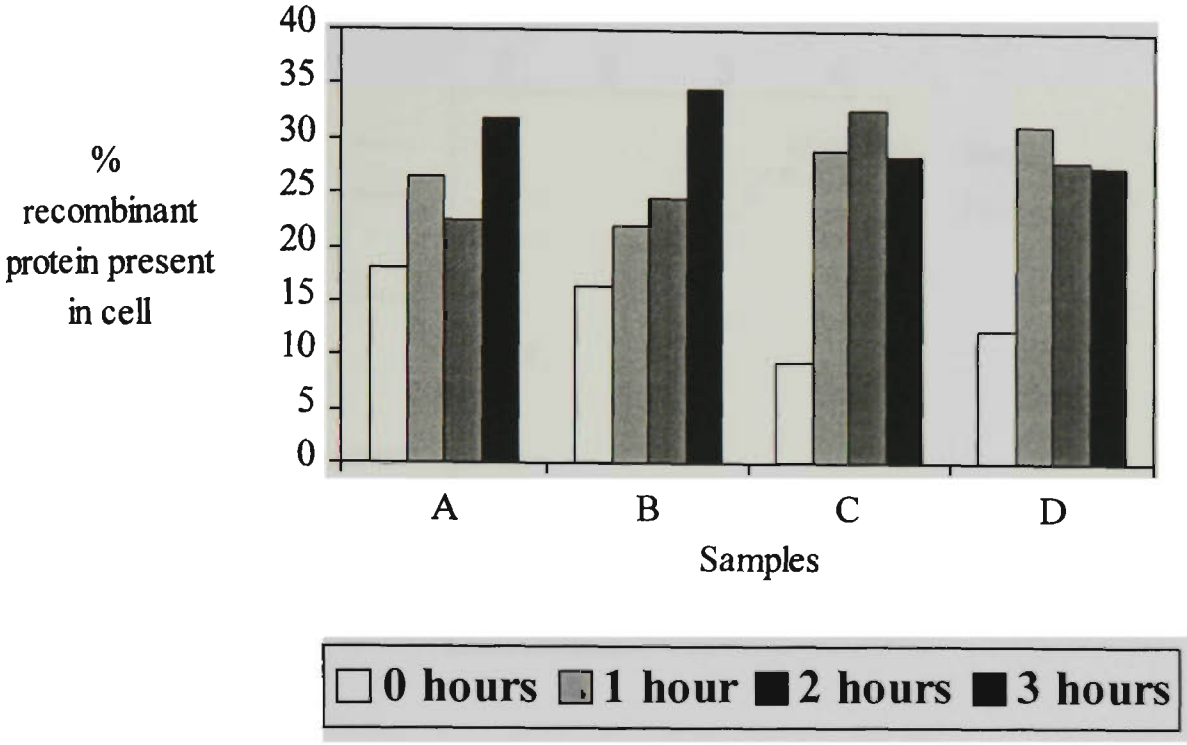


Figure 4.3 Scan profile of SDS-PAGE gel loaded with induced proteins. SDS-polyacrylamide gels containing the protein bands as described in figs. 4.3A and 4.3B were scanned using a Bio-Rad densitometer. The scans were profiled by Molecular Analyst software. Peaks corresponding to the induced proteins were further profiled to approximate percentage-induced protein present in the loaded sample. Figure shows the percentage induced protein present when *E. coli* HMS147(DE3) expressing recombinant N-apxIV was induced with 0.5 mM IPTG (**A**), 2.0 mM IPTG(**B**) and *E. coli* HMS147(DE3) expressing recombinant C-apxIV was induced with 0.5 mM IPTG (**C**), and 2.0 mM IPTG(**D**).

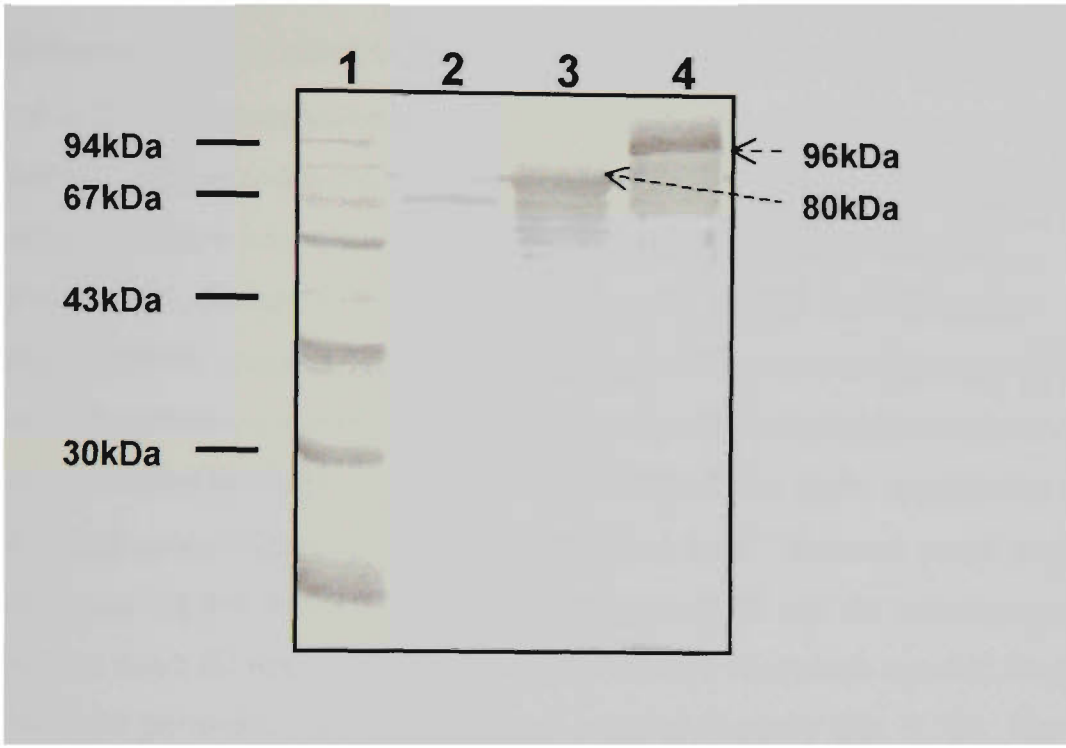


Figure 4.4 Immunoblot depicting the reactivity of pooled sera from pigs challenged with *A. pleuropneumoniae* Sv 7(WF83) against an antigen panel consisting of *A. pleuropneumoniae* HS54, *E. coli* JM109λnir/pQE9 (host/plasmid control), *E. coli* HMS147(DE3)/pJFFapxIVA3Chis (C-ApxIV), and *E. coli* HMS147(DE3)/pJFFapxIV1His2 (N-ApxIV) and (labeled 1, 2, 3 and 4 respectively). For SDS-PAGE, the equivalent of 5 μg protein was loaded onto each lane of a 12% polyacrylamide SDS gel with a 4% stacking gel and the gel run under denaturing conditions (Laemmli, 1970). The proteins were transferred electrophorically onto nitrocellulose. Pooled sera were added at a dilution of 1/500 for 90 minutes. Antibodies were detected by the addition of goat anti pig serum at a dilution of 1/1000. The immunoreactivity was visualized by the addition of 50 μl 2,2-azino bis (3 ethylbenzthiazoline-6-sulphonic acid) diammonium salt (ABTS). Arrows indicate the positions of immunoreactive bands corresponding to the apparent molecular sizes of the recombinant toxin antigens (Lanes 3 and 4).

4.3.5 Assessment of immunoreactivity of recombinant C-apxIV and N-apxIV against sera from infected pigs

To assess their utility as serological reagents, purified recombinant C-apxIV and N-apxIV antigens were coupled to ELISA plates at a concentration of 2 μg/ml (see Section 4.3.2). The immunoreactivity of the purified recombinant antigens was assessed by reacting the antigen against sera from 12 randomly selected healthy piglets that had been brought into a commercial piggery with endemic pleuropneumonia. Serum was collected at days 28, 60, 92 and 128 after introduction into the piggery (fig. 4.7). For comparison, ApxI antigen (purified from culture supernatants of nutrient-shocked cultures of *A. pleuropneumoniae* serovar I) was also coupled to ELISA plates at 2 μg/ml and these were assayed in parallel with the ApxIV recombinant antigens. The data in fig 4.7 showed weak antibody responses against the recombinant antigens at days 28 and 60 with increased responses at days 92 and 128 (fig. 4.7A,B). Immune response against C-apxIV (fig. 4.7A) was generally higher than those against N-apxIV (fig. 4.7B). The immune response against hemolysin was more enhanced, being strong at day 28, and increasing with time until day 92 (fig. 4.7C). One of the most important pieces of information to deduce from this comparative ELISA data, is the differential responses registered by individual pigs against ApxI, C-apxIV and N-apxIV antigens. The individual pig responses are summarized in Table 4.3 where it can be seen that while most animals responded well to ApxI, the antibody response against C and N antigens varied between animals.

Table 4.3. Differential reactivity of pig sera against recombinant C-ApxIV, N-ApxIV and Apx1 (hemolysin)(L= ELISA reactivity between 0.2 and 0.6; H= ELISA reactivity higher than 0.6; N= ELISA reactivity less than 0.2)

Pig number	Against C-ApxIV	Against N-ApxIV	Against hemolysin
1	L	L	H
2	H	L	H
3	H	H	H
4	H	H	H
5	H	L	H
6	N	N	H
7	H	N	H
8	N	N	H
9	H	H	H
10	L	H	H
11	N	N	H
12	N	N	H

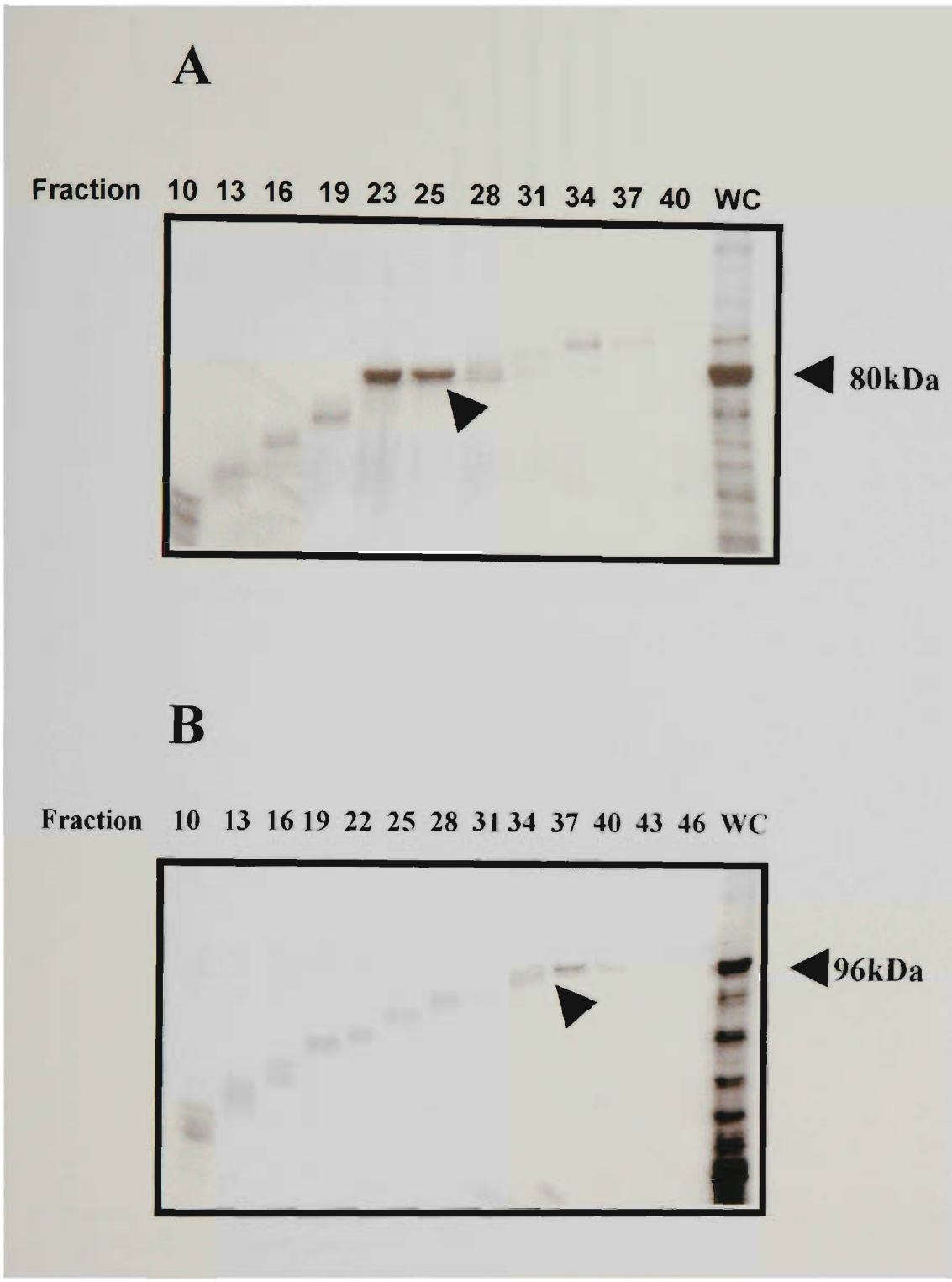
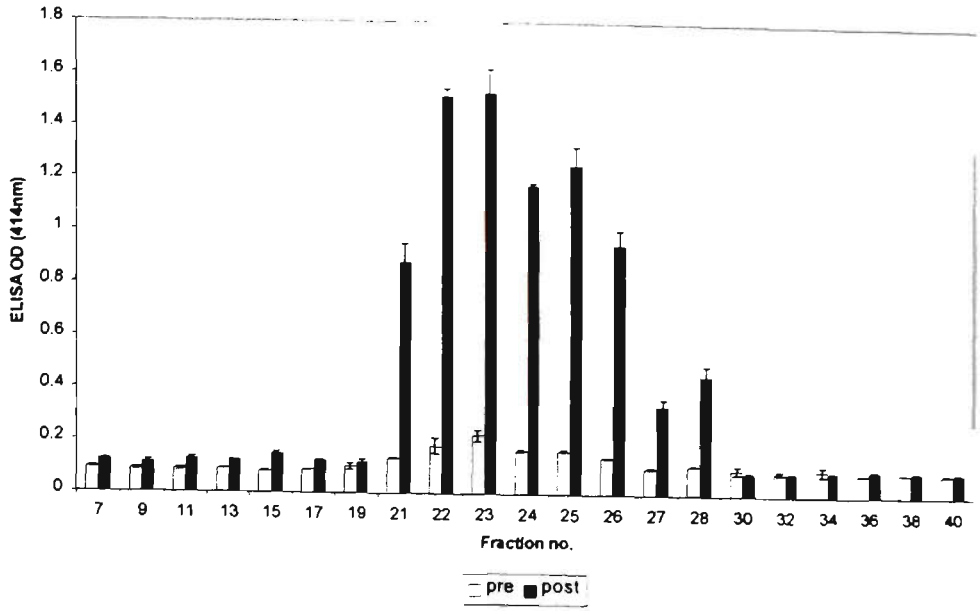
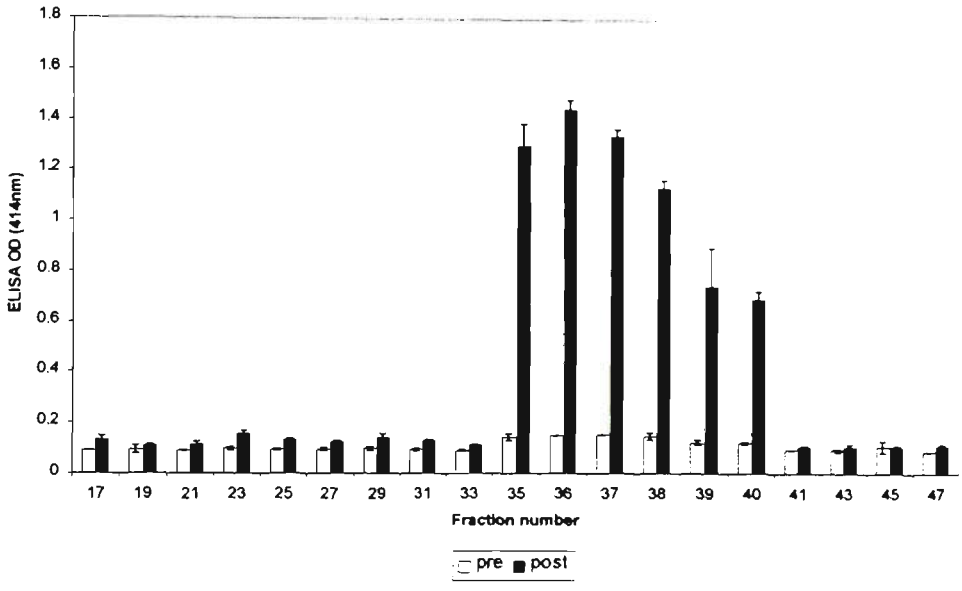


Figure 4.5 Resolution of subunit antigens of *E. coli* HMS147(DE3) expressing (**A**) recombinant N-apxIV and (**B**) recombinant C-apxIV by SDS-PAGE gel. *E. coli* HMS147(DE3) containing the recombinant plasmids was induced with 2 mM IPTG and samples taken at 3 hours after induction. The samples (100 mg) were resuspended in 1 ml Laemmli's reducing mix and loaded onto 6% acrylamide preparative SDS-PAGE column. Every third fraction was run on an 8% SDS polyacrylamide gel and visualized by silver stain. Fraction numbers are indicated on top of each lane. WC represents recombinant host *E. coli* whole cell extract. Arrowheads indicate the position of the recombinant proteins.



(A)



(B)

Figure 4.6 Identification of recombinant subunit antigens by ELISA. (**A**) ELISA profile of host *E. coli* HMS147(DE3) to identify the recombinant N-apxIV in fractions collected after preparative SDS-PAGE. Selected fractions were diluted to 2 μg/ml and coupled to ELISA plates. Immunoreactivity was detected by standard ELISA using swine anti-*A. pleuropneumoniae* sera. Swine IgG that reacted with the recombinant N-apxIV was detected with HRP-conjugated goat anti-swine IgG. (**B**) ELISA profile of host *E. coli* HMS147 (DE3) to locate the recombinant C-apxIV in fractions collected after preparative SDS-PAGE. Selected fractions were coupled to ELISA plates after diluting to 2 μg/ml. Immunoreactivity was detected by standard ELISA using swine anti-*A. pleuropneumoniae* sera. Swine IgG that reacted with the recombinant C-apxIV was detected with HRP-conjugated goat anti-swine IgG. Serum from animals prior to challenge is labeled "pre" while serum obtained from animals after challenge with *A. pleuropneumoniae* labeled "post".

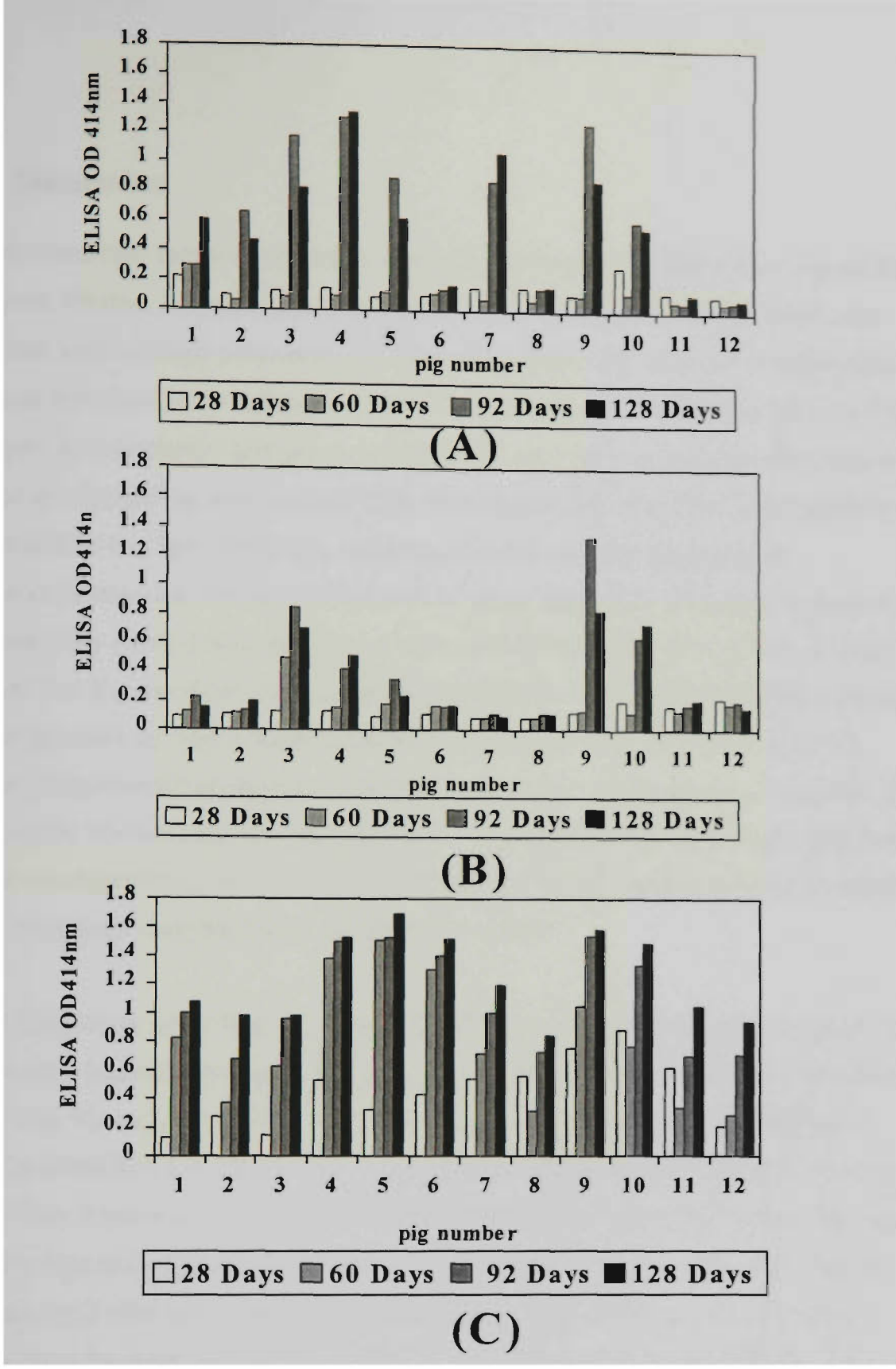


Figure 4.7 Antibody response against (**A**) recombinant C-apxIV, (**B**) recombinant N-apxIV and (**C**) *A. pleuropneumoniae* hemolysin (ApxI) in exposed pigs in piggery with endemic pneumonia at day 28, day 60, day 92, and day 125 as assessed by standard ELISA. Preparative SDS-PAGE purified recombinant N-apxIV (2μg/ml) and recombinant C-apxIV (2μg/ml) (section 4.3.3), as well as purified hemolysin (2μg/ml), were coupled to ELISA plates as described in methods. The antigens were then reacted against sera from pigs from a piggery with endemic pneumonia. Sera that reacted with the antigens were then detected with HRP-conjugated goat anti-swine IgG. (I would like to acknowledge the inputs of Dr. James Chin for providing the sera from the piggery and to Bernadette Turner for contributing to some of the ELISA experiments).

4.4 Discussion

There were two factors that made it feasible to evaluate ApxIV toxin as an ELISA reagent. Firstly, Schaller *et al* were able to demonstrate that sera from pigs infected with various serovars of *A. pleuropneumoniae*, reacted in immunoblots against the rAp4 toxin antigen. Therefore the ApxIV toxin, like the 39 kDa OMP antigen, is a common antigen and will detect sero-converted pigs that may have become infected by any serovar of *A. pleuropneumoniae*. This is particularly important in the field since the isolation, culture and serotyping of *A. pleuropneumoniae* can be difficult and tedious. Secondly, Schaller *et al* had successfully cloned both the C and N-terminal fragments of ApxIV in *E. coli*. This meant that the purification of C-ApxIV (96kDa) and N-apxIV (80 kDa) could be accomplished by preparative SDS-PAGE without introducing the risk of *A. pleuropneumoniae* antigens contaminating the purified fractions. However, it was necessary to demonstrate also that subunit antigens of *E. coli* origin with molecular sizes corresponding with 96 and 80 kDa respectively, were not reactive against sera from pigs exposed to *A. pleuropneumoniae*.

PCR fragments encoding the C and N-terminal coding sequences of *apxIV* had previously been cloned by Schaller *et al.* (1999) into a derivative pET plasmid (pET14b, Novagen; fig. 4.8) to yield pJFFapxIVA1NaHis1 (N-terminal) and pJFFapxIVA3Chis (C-terminal) respectively. These plasmids were sent to Dr. J. Chin from Professor J. Frey as part of a collaborative program to develop an ApxIV ELISA. Recombinant *E. coli* encoding these plasmids was cultured in media containing 2 mM IPTG (isopropyl-β-D-thiogalactopyranoside) to induce the production of rAp4. As shown in fig. 4.1, addition of IPTG arrested the growth of both recombinant clones with concomitant production of C-ApxIV (96 kDa) and N-ApxIV (80 kDa) antigens respectively (fig. 4.2).

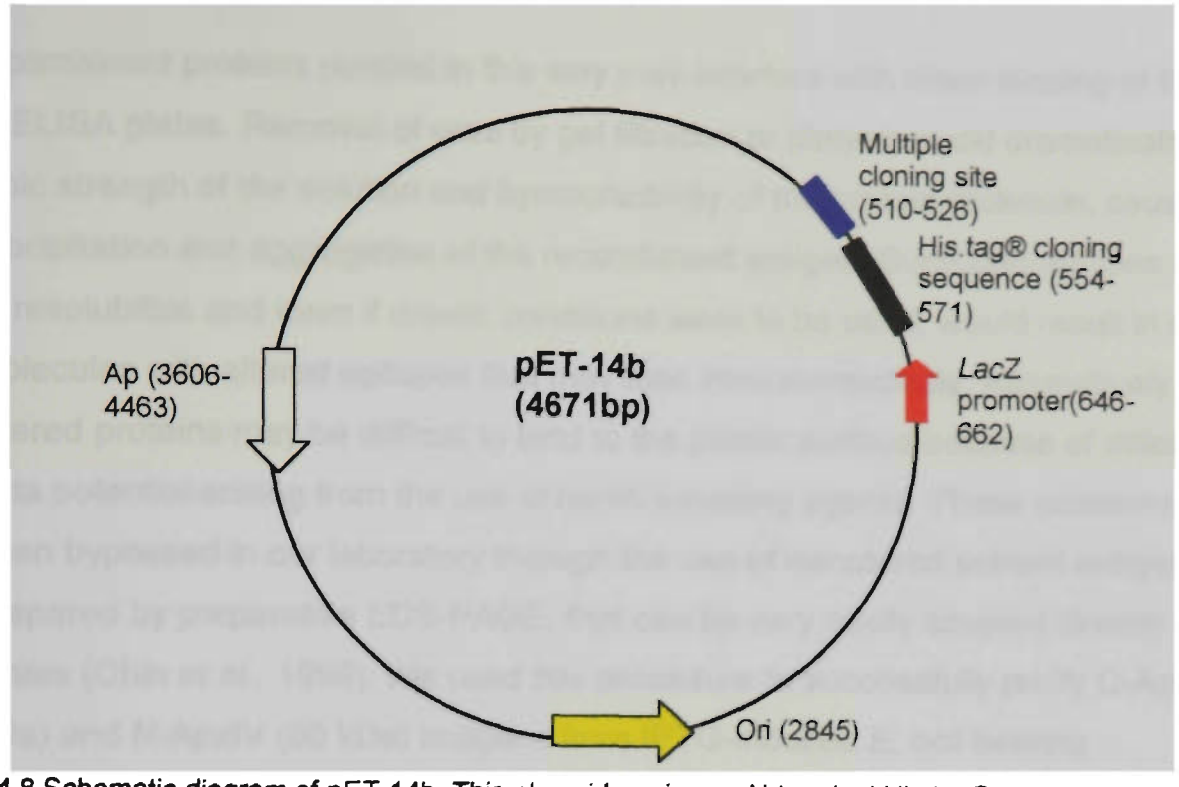


fig. 4.8 Schematic diagram of pET-14b. This plasmid carries an N-terminal His-tag® sequence followed by a thrombin site and three cloning sites (*Nde*I, *Xho*I, *Bam*HI). Target genes are cloned in pET plasmids under the control of *lacZ* promoter. Presence of *bla* coding sequence (3306-4463) confers ampicillin (Ap) resistance (arrow). Origin of replication (OriR) is present at 2845bp. (Adapted from Novagen pET manual, 1998).

The immunoreactivity of C-ApxIV (96 kDa) and N-ApxIV (80 kDa) antigens were confirmed in immunoblots using pooled sera from pigs challenged with *A. pleuropneumoniae* serovar 1. The data in fig. 4.4 show that both recombinant antigens (lanes 3 and 4) were immunoreactive whilst antigens from the *E. coli* host (lanes 1 and 2) were not. These results confirmed Schaller's recently published data (1999) and permitted the purification of the two recombinant antigens by preparative SDS-PAGE.

The main reason for using a pET plasmid vector was to clone both the C-terminal and N-terminal fragments of *apxIV* in frame with the his-tag site. This would allow for the expression of fusion C or N-terminal ApxIV protein fragments bearing a C-terminal histidine decamer and an N-terminal histidine hexamer respectively. Such his-tagged fusion proteins can be purified by Ni-NTA chromatography. However, this protocol was not used for the generation of purified C-ApxIV (96 kDa) and N-ApxIV (80 kDa) antigens because of the need to elute his-tagged proteins from Ni affinity gels with high concentrations of urea at a reduced pH. The residual urea content present in

recombinant proteins purified in this way may interfere with direct binding of the antigen to ELISA plates. Removal of urea by gel filtration or dialysis could dramatically alter the ionic strength of the solution and hydrophobicity of the protein molecule, causing *in situ* precipitation and aggregation of the recombinant antigen. Such preparations are difficult to resolubilize and even if drastic conditions were to be used, would result in solvated molecules with altered epitopes that may lose immunoreacitvity. Alternatively, these altered proteins may be difficult to bind to the plastic surface because of differences in zeta potential arising from the use of harsh solvating agents. These problems have been bypassed in our laboratory through the use of denatured subunit antigens prepared by preparative SDS-PAGE, that can be very easily coupled directly to ELISA plates (Chin *et al.*, 1996). We used this procedure to successfully purify C-ApxIV (96 kDa) and N-ApxIV (80 kDa) antigens from IPTG-induced *E. coli* bearing pJFFapxIVA3Chis (C-terminal) and pJFFapxIVA1NaHis1 (N-terminal) plasmids respectively (figs. 4.5 and 4.6).

Fig. 4.7 shows the temporal ELISA response of pigs from a piggery with endemic pleuropneumonia against SDS-PAGE purified C-ApxIV (fig. 4.7A) and N-ApxIV (fig. 4.7B) antigens. For comparison, their responses against ApxI toxin are also included (fig. 4.7C). Pigs 6, 8 and 12 showed no immunoreactivity against either C-ApxIV or N-ApxIV antigen but reacted moderately well against ApxI (Table 4.3). This demonstrated clearly that there are no cross-reactivities between antibodies directed against ApxIV and that of ApxI. ApxI is constitutively expressed by *A. pleuropneumoniae* very early in infection. It is therefore not surprising that bloods from all pigs were immunoreactive by 60 d in comparison to a complete lack of reactivity at this time point against either C-ApxIV or N-ApxIV antigen. The delayed serological reactivity against ApxIV compared to ApxI may be associated with continued proliferation of the pathogen *in vivo* and sustained ApxIV antigen production in a successful clinical infection as compared to an abortive infection where early colonization by the pathogen may have been arrested by immunological responses. Another notable feature was the higher level of reactivity seen in 8 out of 12 pigs against the C-ApxIV antigen compared to 4 out of 12 pigs for the N-ApxIV antigen. It is likely from a consideration of the Apx toxin structure (fig. 1.1)

that insertion of the N-terminal end within the target cell shields this epitope from the immune system whilst the C-terminal, which protrudes from the cell, is highly "visible" to the immune system. Consequently, there may be a greater disposition to generate antibodies against C-terminal compared to N-terminal epitopes.

The use of recombinant C-ApxIV and N-ApxIV as possible ELISA reagents have shown promising results. This is the very first time that this recombinant toxin antigen has been trialed as a possible ELISA reagent, and therefore there are undoubtedly many issues that have not been addressed. The issues of sensitivity as well as species-specificity have not been looked at, although results from this work did show the absence of cross- reactivity of the recombinant toxins against pig sera positive to ApxI and vice-versa (fig. 4.7). It would be interesting to observe how the recombinant ApxIV toxin cloned in its entirety would react in an ELISA as compared to C-ApxIV and N-ApxIV. Further work needs to be done to answer those questions. This work has laid a foundation for future work to follow.

Chapter 5: Conclusion

The major conclusions arising from this work are summarized below:

The Preparative SDS-PAGE technology is extremely useful for the purification of both outer membrane proteins and exocellular toxins of *A. pleuropneumoniae*. Denatured antigens generated in this way can be used directly for coupling to ELISA plates. Such antigens can be easily evaluated as ELISA reagents. The 39 kDa OMP of *A. pleuropneumoniae* as well as the recombinant ApxIV toxin domains were successfully purified and evaluated as ELISA reagents.

The 39 kDa OMP of *A. pleuropneumoniae* was demonstrated to be highly immunoreactive in an ELISA test and can be used to monitor temporal serological responses of pigs following experimental infection with *A. pleuropneumoniae*. The precision, specificity and sensitivity of this antigen is currently being assessed.

The recombinant ApxIV toxin antigens appear to be of value in predicting the clinical status of *A. pleuropneumoniae* infected pigs. A correlation between ELISA responses against either the C-ApxIV and N-ApxIV antigen and the severity of lung pneumonia can be established once necropsies have been carried out on both clinically affected and subclinical pigs in conjunction with serological data based on the two recombinant antigens.

Chapter 6: References

Anderson, C., Potter, A.A., and G.F. Gerlach. 1991. Isolation and molecular characterization of spontaneously occurring cytolysin-negative mutants of *Actinobacillus pleuropneumoniae*. Infect. Immun. 59: 4110-4116

Anderson, T.J. and J.I. McInnes.1997. Expression and phylogenetic relationships of a novel lacZ homologue from *Actinobacillus pleuropneumoniae*. FEMS Microbiol. Lett. 152: 117-123

Austin, B. and F.G. Priest. 1986. Modern Bacterial Taxonomy. United Kingdom: Van Nostrand Reinhold.

Beck, M., Van Den Bosch, J.F., Jongenelen, I.M.C.A., Loeffen, P.L.W., Nielsen, R., Nicolet, J. and J. Frey. 1994. RTX toxin genotypes and phenotypes in *Actinobacillus pleuropneumoniae* field strains. J. Clin. Microbiol. 32: 2749-2754

Bendixen, P.H., Shewen, P.E., Rosendal, S. and B.N. Wilkie. 1981. Toxicity of *Haemophilus pleuropneumoniae* for porcine lung macrophages, peripheral blood monocytes, and testicular cells. Infect. Immun. 33: 673-676

Blackall, P.J. and J.L. Pahoff. 1995. Characterization of porcine haemophili isolated from Australian pigs between 1988 and 1992. Aust. Vet. J. 72: 18-21

Blanchard, P.C., Walker, R.L. and I. Gardner. 1993. Pleuropneumonia in swine associated with a urease-negative variant of *Actinobacillus pleuropneumoniae* serotype 1. J. Vet. Diagn. Invest. 4: 270-278

Bloom, H., Beier, H., and H.S. Gross. 1987. Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. Electrophoresis. 8: 93-99

Bosse, J.T., Johnson, R.P. and S. Rosendal. 1989. Capsular polysaccharide antigens for detection of serotype-specific antibodies to *Actinobacillus pleuropneumoniae*. Can. J. Vet. Res. 54: 320-325

Bunka, S., Christensen, C., Potter, A.A., Willson, P.J. and G-F. Gerlach. 1995. Cloning and characterization of a protective outer membrane lipoprotein of *Actinobacillus pleuropneumoniae* serotype 5. Infect. Immun. 63: 2797-2800

Burrows, L.L. and R.Y.C. Lo. 1992. Molecular characterization of an RTX toxin determinant from *Actinobacillus suis*. Infect. Immun. 60: 2166-2173

Chin, J. and Y. Dai. 1990. Selective extraction of outer membrane proteins from membrane complexes of *Pseudomonas maltophila* by chloroform–methanol. Vet. Microbiol. 22: 69-78

Chin, J., Djordjevic, S., Scarman, A. and R. Petersen. 1996. Subunit antigen hunting using Preparative SDS-PAGE Profiling (PPP) technology. Bio-Rad US/EG Bulletin Application Note.

Chin, J. and B. Turner. 1990. Extraction of membrane antigens from *Brucella ovis* and an assessment of their serological activity by immunoblotting. J. Gen. Microbiol. 136: 1615-1622

Chin, J.C., Dai, Y. and J.E. Watts. 1995. Antibody response against *Pseudomonas aeruginosa* membrane proteins in experimentally infected sheep. Vet. Microbiol. 43: 21-32

Chin, J.C. and C. Scully. 1986. Identification of immunoreactive antigens of *Brucella ovis* by ELISA profiling. Res. Vet. Sci. 41: 1-6

Costerton, J.W., Irvin, R.T. and K.J. Cheng.1981. The role of bacterial surface structures in pathogenesis. Crit. Rev. Microbiol. 8:303-383

Cruz, W.T., Nedialkov, Y.A., Thacker, B.J. and M.H. Mulks. 1996. Molecular characterization of a common 48-Kilodalton outer membrane protein of *Actinobacillus pleuropneumoniae.* Infect. Immun. 64: 83-90

Deneer, H.G. and A. Potter. 1989. Effect of Iron restriction on the outer membrane proteins of *Actinobacillus (Haemophilus) pleuropneumoniae*. Infect. Immun. 57: 798-804

Devenish, J., Rosendal, S., Bosse, J.T. and R. Johnson. 1990. Prevalence of seroreactors to the 104-kilodalton hemolysin of Actinobacillus pleuropneumoniae in swine herds. J. Clin. Microbiol. 28: 789-791

Dom, P., Haesebrouck, F., Ducatelle, R. and G. Charlier. 1994. *In vivo* association of *Actinobacillus pleuropneumoniae* serotype 2 with the respiratory epithelium of pigs. Infect. Immun. 62: 1262-1267

Fedorka-Cray,P.J., Hoffman,L., Cray,W.C., Gray,J.T., Breisch,S.A. and G.A. Anderson. 1993. *Actinobacillus pleuropneumoniae*. Part I. History, epidemiology, serotyping, and treatment. 1993. The Compendium. 15(10): 1447-1453.

Fedorka-Cray,P.J., Hoffman,L., Cray,W.C., Gray,J.T., Breisch,S.A. and G.A. Anderson. 1993. *Actinobacillus pleuropneumoniae*. Part II. Virulence factors, immunity, and vaccines. 1993. The Compendium. 16(1): 117-123.

Fedorka-Cray, P.J., Stine D.L., Greenwald, J.M., Gray, J.T., Huether, M.J., and G.A. Anderson. 1994. The importance of secreted virulence factors in *Actinobacillus pleuropneumoniae* bacterin preparation: a comparison. Vet.Microbiol. 37: 85-100

Fenwick, B. and S. Henry. 1994. Porcine pleuropneumonia. J. Am.Vet.Med. Assoc. 204: 1334-1340

Filip, C., Fletcher, G., Wulff, J.L. and C.F. Earhart. 1973. Solubilization of the cytoplasmic membrane of *Escherichia coli* by the ionic detergent sodium-lauryl sarcosinate. J. Bacteriol. 115: 717-722

Fodor, L., Varga, J., Molnar, E. and I. Hajtos. 1989. Biochemical and serological characterization of *Actinobacillus pleuropneumoniae* biotype 2 strains isolated from swine. Vet. Microbiol. 20: 173-180

Frank, R.K., Chengappa, M.M. and R.D. Oberst. 1992. Pleuropneumonia caused by *Actinobacillus pleuropneumoniae* biotype 2 in growing and finishing pigs. J. Vet. Diagn. Invest. 4: 270-278

Frey, J. Exotoxins of *Actinobacillus pleuropneumoniae.* 1995. In: Donachie, W., Lainson, F.A., Hodgson, J.C. (eds). *Haemophilus*, *Actinobacillus*, and *Pasteurella.* Plenum, New York and London pp 101-113

Frey, J., Bosse, J.T., Chang, Y.-F., Cullen, J.M., Fenwick, B., Gerlach, G.F., Gygi, D., Haesebrouck, F., Inzana, T.J., Jansen, R., Kampl, E.M., Macdonald, J., AmacInnes, J.I., Mittal, K.R., Nicolet, J., Rycroft, A., Segers, R.P.A.M., Smits, M.A., Stenbaek, E., Struck, D.K., Van den Bosch, J.F., Willson, P.J. and R. Young. 1993. *Actinobacillus pleuropneumoniae*-RTX toxins: uniform designation of haemolysins, cytolysins, pleurotoxin and their genes. J. Gen. Microbiol. 139: 1723-1728.

Frey, J., Kuhnert, P., Villiger, L. and J. Nicolet.. 1996. Cloning and characterization of an *Actinobacillus pleuropneumoniae* outer membrane protein belonging to the family of PAL lipoproteins. Res. Microbiol. 147: 351-361

Gardner, K. 1996. Immunological characteristics of *A. pleuropneumoniae.* Honours Theses. University of Technology Sydney.

Gerlach, G-F., Anderson, C., Klashinsky, S., Rossi-Campos, A., Potter, A.A. and P.J. Willson. 1993. Molecular characterization of a protective outer membrane lipoprotein (OmlA) from *Actinobacillus pleuropneumoniae* serotype 1. Infect. Immun. 61: 565-572

Gottschalk, M., DeLasalle, F., Radaacovici, S. and J.D. Dubreuil. 1994. Evaluation of long chain lipopolysaccharides (LC-LPS) of *Actinobacillus pleuropneumoniae* serotype 5 for the serodiagnosis of swine pleuropneumonia. Vet. Microbiol. 38: 315-327

Haesebrouck, F., Chiers, K., Van Overbeke, I. and R. Ducatelle. 1997. *Actinobacillus pleuropneumoniae* infection in pigs: the role of virulence factors in pathogenesis and protection. Vet. Microbiol. 58: 239-249

Holland, J., Parsons, T.R., Hasan, A.A., Cook, S.M., Stevenson, P., Griffiths, E. and P. Williams. 1996. Conservation and antigenic cross-reactivity of the transferrin- binding proteins of *Haemophilus influenzae*, *Actinobacillus pleuropneumoniae* and *Neisseria meningitidis*. Microbiol. 142: 3505-3513

Inzana, T.J. 1991. Virulence properties of *Actinobacillus pleuropneumoniae*. Microb. Path. 11: 305-316

Ito, H., Uchida, I., Sekizaki, T., Ooishi, E., Kawai, T., Okabe, T., Taneno, A. and N. Terakado. 1995. Molecular cloning of an *Actinobacillus pleuropneumoniae* outer membrane lipoprotein (Oml A) from serotype 5a. Microb. Path. 18: 29-36

Jansen, R. 1994. The RTX toxins of *Actinobacillus pleuropneumoniae*. PhD thesis. Utrecht, the Netherlands.

Jansen, R., Briaire, J., Kamp, E.M., Gielkens, A.L. and M.A. Smits. 1993. Structural analysis of the *Actinobacillus pleuropneumoniae*-RTX-toxinI (ApxI) operon. Infect. Immun. 61: 3688-3695

Jansen, R., Briaire, J., Van Geel, A.B.M., Kamp, E.M., Gielkens, A.L. and M.A. Smits. 1994. Genetic map of the Actinobacillus pleuropneumoniae RTX-toxin (Apx) operons: Characterization of the ApxIII operons. Infect. Immun. 62: 4411-4418

Jensen, A.E. and T.A. Bertram. 1986. Morphological and biochemical comparison of virulent and avirulent isolates of *Haemophilus pleuropneumoniae* serotype 5. Infect. Immun. 51: 419-424

Jolie, R.A.V., Mulks, M.H. and B.J. Thacker. 1994. Antigenic differences within *Actinobacillus pleuropneumoniae* serotype 1. J. Vet. Microbiol. 38: 329-349

Kamp, E.M., Popma, J.K., Anakotta, J. and M.A. Smits.1991. Identification of hemolytic and cytotoxic proteins of *Actinobacillus pleuropneumoniae* by use of monoclonal antibodies. Infect. Immun. 59: 3079-3085

Laemmli, U.D. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage. Nature. 227: 680-685

Lu, Y.S., Afendis, S. and S.P. Pakes. 1988. Identification of immunogenic outer membrane proteins of *Pasteurella multocida* in rabbits. Infect. Immun. 56: 1532-1537

Ma, J. and T.Inzana, 1990. Indirect enzyme linked immunosorbent assay for detection of antibody to 110,000-molecular weight hemolysin of *Actinobacillus pleuropneumoniae.* J. Clin. Microbiol. 28: 1356-1361

MacDonald, J. and A.N. Rycroft. 1992. Molecular cloning and expression of ptxA, the gene encoding the 120 kDa cytotoxin of *Actinobacillus pleuropneumoniae* serotype 2. Infect. Immun. 60: 2726-2732

MacInnes, J.I. and Janice D. Borr. 1990. The family *Pasteurellaceae*: modern approaches to taxonomy. Can. J. Vet. Res. 54: S6-S11

MacInnes, J.I. and S. Rosendal. 1987. Analysis of major antigens of *Haemophilus (Actinobacillus) pleuropneumoniae* and related organisms. Infect. Immun. 55: 1626-1630.

Madsen, M.E., Carnahan, K.G. and R.N. Thwaites. 1995. Evaluation of pig lungs following an experimental challenge with *Actinobacillus pleuropneumoniae* serotype 1 and 5 in pigs inoculated with either hemolysin protein and /or outer membrane proteins. FEMS Microbiol. Lett. 131: 329-335

Mannheim, W. Family III *Pasteurellaceae*. Pohl 1981a, 382. In Krieg N.R., Holt, J.G., eds. Bergey's Manual of Systemic Bacteriology. Baltimore: Williams and Wilkins, 1984.

Mosier, D.A., Simons, K.R., Confer,A.W., Panciera, R.J. and K.D. Clinkenbeard. 1989. *Pasteurella haemolytica* antigens associated with resistance to pneumonic pasteruellosis. Infect. Immun. 57: 711-716

Mylrea, P.J., Fraser, G., Macqeen, P. and D.A. Lambourne. 1974. Pleuropneumonia in pigs caused by Haemophilus parahaemolyticus. Aust. Vet. J. 50: 255-259

Nicolet, J. Overview of the virulence attributes of the HAP-group of bacteria. 1990. Can.J.Vet.Res. 54: S12-S14

Nicolet, J. *Actinobacillus pleuropneumoniae*. 1992. In Leman, A.D., Straw, B., Mengelin, W.M., D'Allaire, S., Taylor, D.J., (Eds.) Diseases of Swine. Ames, Iowa State University Press, pp 401-408.

Nicolet, J. and E. Scholl. *Haemophilus* infections. 1981. In Leman, A.D., Glock, R.D., Mengelin, Penny, R.H.C, Scholl, E., and Straw, B. Diseases of Swine. 5th Ed. Iowa State University Press, Ames, pp 368-377

Nielsen, R. Serological characterization of *Actinobacillus pleuropneumoniae* strains and proposal of a new serotype: serotype 12. Acta. Vet. Scand. 27: 453-455

Noyes, E.P., Feeney, D.A. and C.Pijoan. 1990. Comparison of the effect of pneumonia detected during lifetime with pneumonia detected at slaughter on growth in swine. J. Am. Vet. Med. Assoc. 197: 1025-1029

Pohl, S., Bertschinger, H.U., Frederiksen, W. and W.Mannheim.1983. Transfer of *Haemophilus pleuropneumoniae* and the *Pasteurella haemolytica*-like organism causing porcine necrotic pleuropneumoniae to the genus *Actinobacillus* (*Actinobacillus pleuropneumoniae* comb.nov) on the basis of phenotypic and deoxyribonucleic acid relatedness. Int. J. Syst. Bacteriol. 33(3): 510-515

Pointon, A.M., Skirrow, S.Z. Moore, M.J., Cargill, C.F., Poomvises, P. and P. Ingkaninun. 1995. Monitoring disease trends in the Australian pig. Proceedings: The 13th International Pig Veterinary Society Congress, Bangkok, Thailand, 26-30 June 1994. Faculty of Veterinary Science, Chulalongkorn University; Bangkok; Thailand.

Radacovici, S., Gottschalk, M. and J.D. Dubreuil. 1994. Lipopolysaccharides of *Actinobacillus pleuropneumoniae* (serotype 1): a readily obtainable antigen for ELISA serodiagnosis of pig pleuropneumoniae. Vet. Microbiol. 39: 219-230

Rapp, V.J., Munson, R.S., Jr. and R.F. Ross. 1986. Outer membrane protein profiles of *Haemophilus pleuropneumoniae.* Infect. Immun. 52: 414-42

Rapp, V.J. and R.F. Ross. 1986. Antibody response of swine to outer membrane components of *Haemophilus pleuropneumoniae* during infection. Infect. Immun. 54: 751-760

Rosendal, S., Devenish, J., MacInnes, J.I., Lumsden, J.H., Watson, S. and H. Xun. 1988. Evaluation of heat sensitive, neutrophil-toxic, and hemolytic activity of *Hemophilus (Actinobacillus) pleuropneumoniae.* Am. J. Vet. Res. 49: 1053-1058

Schaller, A., Kuhn, R., Kuhnert, P., Nicolet, J., Anderson, T.J., MacInnes, J.I., Segers, R.P.A.M. and J. Frey. 1999. Characterization of apxIVA, a new RTX determinant of *Actinobacillus pleuropneumoniae.* Microbiol. 145: 2105-2116

Sirois, M., Lemire, E.G. and R.C. Levesque. 1991. Construction of a DNA probe and detection of *Actinobacillus pleuropneumoniae* by using polymerase chain reaction. Infect. Immun. 29: 1183-1187

Stanley, P., Packman, L.C., Koronakis, V. and C. Hughes. 1994. Fatty acylation of two internal lysine residues required for the toxic activity of Escherichia coli hemolysin. Science. 266: 1992-1996

Stanley, P., Koronakis, V. and C. Hughes. 1998. Acylation of *Escherichia coli* hemolysin: A unique protein lipidation mechanism underlying toxin function. Microbiol. Mol. Biol. Rev. 62: 309-353

Straw, B.E., Shin, S.J. and Y.E. Yeager. 1990. Effect of pneumonia in growth rate and feed efficiency of minimal disease pigs exposed to *Actinobacillus pleuropneumoniae* and *Mycoplasma hyopneumoniae*. Prev. Vet. Med. 9:287-294

Tarigan, S., Slocombe, R.F., Browning, G.F. and P.J. Blackall. 1996. Characterization of hemolytic RTX toxins produced by Australian isolates of *Actinobacillus pleuropneumoniae*. Aust. Vet. J. 73: 164-169

Thompson, S.A., Wang, L.L., West, A. and P.F. Sparling. 1994. *Neisseria meningitidis* produces iron-regulated proteins related to the RTX family of exoproteins. J. Bacteriol. 175: 811-818

Thwaites, R.N. and S. Kadis.1991. Immunogenicity *of Actinobacillus pleuropneumoniae* Outer Membrane Proteins and enhancement of phagocytosis by antibodies to the proteins. Infect. Immun. 59: 544-549

Towbin, H., Staehlin, T. and J. Gordon. 1979. Electrophoretic transfer of protein from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. U.S.A. 76: 4350-4353

Tsai, C.M. and C.E. Frasch. 1982. A sensitive silver stain for detecting lipopolysaccharide in polyacrylamide gels. Anal. Biochem. 119: 115-119

Van leengoed, L.A., Kamp, E.M. and J.M.A. Pol.1989. Toxicity of *Hemophilus pleuropneumoniae* to porcine lung macrophhages. Vet. Microbiol. 5: 521-528

Wannemuehler, M.J. and J.E. Galvin. 1994. Bacterial immunogens and protective immunity in swine. Vet. Immunol. Immunopath. 43: 117-126

Yanisch-Perron, C., Viera, J. and J. Messing. 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequence of the M13mp18 and pUC19 vectors. Gene. 33: 103-119

Zinnemann, K. Some historical aspects of the genera *Haemophilus*, *Pasteurella* and *Actinobacillus*, and of some of their species. In: Kilian, M., Frederiksen, W., Beberstein, E.L., eds. *Haemophilus*, *Pasteurella* and *Actinobacillus*. New York: Academic Press. 1981: 1-10