DETECTION OF *ACTINOBACILLUS PLEUROPNEUMONIAE* AND IDENTIFICATION OF SEROTYPES 1, 2, AND 8 BY MULTIPLEX PCR

by

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DETECTION OF *ACTINOBACILLUS PLEUROPNEUMONIAE* AND IDENTIFICATION OF SEROTYPES 1, 2, AND 8 BY MULTIPLEX POLYMERASE CHAIN REACTION

by

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(ABSTRACT)

Traditional immunological assays used to serotype *Actinobacillus pleuropneumoniae* have been problematic due to cross- reactivity between serotypes, particularly serotypes 6 and 8. To avoid these serological cross-reactions, a multiplex PCR assay was developed to detect *A. pleuropneumoniae* and identify serotypes 1, 2, and 8. Primers specific to the conserved capsular polysaccharide export region of *A. pleuropneumoniae* serotype 5 amplified a 880 bp fragment in all serotypes excluding serotype 4 or a 489 bp DNA fragment in all serotypes including serotype 4. Primers specific to the capsular polysaccharide biosynthesis regions of *A. pleuropneumoniae* serotypes 1, 2, and 8 amplified a 1.6 kb, a 1.7 kb, and 970 bp fragment in the respective serotype. This PCR assay detects *A. pleuropneumoniae* and identifies serotypes 1, 2, and 8.

This thesis is dedicated to my parents, Jim and Johanna Schuchert,
and to my husband Chip Murdock.

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TABLE OF CONTENTS

List of Figures

Chapter 2 **Page**

List of Tables

Chapter 2 Page

Literature Review

Actinobacillus pleuropneumoniae

Description and History

Actinobacillus pleuropneumoniae is an economically important swine pathogen. It is the causative agent of swine pleuropneumonia a highly contagious and often fatal respiratory disease. *A. pleuropneumoniae* is a member of the family *Pasteurellaceae*, which includes the genera, *Haemophilus*, *Actinobacillus Manheimeia* and *Pasteurella*. The first *Haemophilus*-like organism was isolated from porcine pneumonic lung lesions in England in 1957 (Pattison et al., 1957). A few years later, a *Haemophilus*-like organism associated with pulmonary lesions was discovered in Argentina and California (Sebunya and Saunders, 1983). The incidence of swine pleuropneumonia has increased dramatically since then and has been frequently found in the United States, Canada, Australia, Europe and Japan (Sebunya and Saunders, 1983; Didier et el., 1984). The name *Haemophilus pleuropneumoniae* was suggested for this bacterium by Shope due to its requirement for nicotinamide adenine dinucleotide (NAD) (Shope, 1964) and Killian et al. (1978) confirmed the use of this name by biochemical and serological characterization of *Haemophilus* strains isolated from swine (Nicolet, 1988). The name *H. parahaemolyticus* has also been used to describe isolates of this bacterium in California and Ireland (Straw et al., 1999). *H. pleuropneumoniae* was transferred to the genus *Actinobacillus*, because DNA-DNA hybridization studies revealed that

H. pleuropneumoniae Shope 4074 was more closely related to the type-species of *A. lignieresii* than to the type-species of the genus *H. influenzae* (Pohl et al., 1983; Borr, 1991; Inui et al., 2000). *A. pleuropneumoniae* is characterized as a gram-negative, encapsulated, facultatively anaerobic, hemolytic, non-spore forming, non-motile, coccobacillus (Pohl et al., 1983; Sebunya and Saunders, 1983). *A. pleuropneumoniae* isolates that require NAD for growth are designated as biovar 1. To date, there are 12 serotypes of biovar 1, which are classified by their unique capsular polysaccharide (Inzana and Mathison, 1997). Serotypes 1 and 5 are further subdivided into 1a and 1b and 5a and 5b based on slight antigenic differences due to polysaccharide structure. *A. pleuropneumoniae* that do not require NAD for growth, but do require specific pyridine nucleotide precursors for NAD synthesis (Niven and Levesque, 1988; Martin et al., 2001) are designated as biovar 2 of which there are currently 6 serotypes (Bosse et al., 2002). A new serovar of *A. pleuropneumoniae*, serovar 15, is currently proposed with HS143 as the reference strain for the new serovar (Blackhall et al., 2002). In general, biovar 1 strains are considered to be more virulent than biovar 2 strains (Gram and Ahrens, 1998).

Transmission

A. pleuropneumoniae is highly contagious and is transmitted mainly by direct contact with an infected pig. *A. pleuropneumoniae* does not survive long in the environment and there are no other natural hosts of the bacterium (MacInnes and Rosendal, 1988; Bosse et al., 2002). The main route of spread is by aerosol and it is commonly spread between herds by carriers, from pen to pen by air handling systems, or

through vehicles and transport drivers (Fussing et al., 1998). The highest risk of transmission of *A. pleuropneumoniae* to piglets is in the farrowing pen (Wongnarkpet et al., 1999). Several factors influence the potential to introduce *A. pleuropneumoniae* into a herd including the frequency and number of swine purchased and where they were purchased from. Addition of swine to an existing pathogen free herd always increases the probability of infection. To reduce this risk, new animals should be quarantined and swine should be purchased from a reputable breeder and from a stock that has been health examined. Introduction of *A. pleuropneumoniae* into a pathogen- free herd could cause morbidity and mortality in pigs of all ages depending on the immune status of the herd. The highest losses may be in feeder/finishing pigs due to a decreased immune status and increased growth rate. During this period, housing conditions have intensified and altered the relationship between the microorganisms, the pigs and their environment (Didier et al., 1984; Ice et al., 1999). For a herd that is already infected, factors such as crowding, sudden climatic changes or ventilation problems influence the possibility of an outbreak. In temperate climates, the incidence of disease increases from a low in the summer to a peak in winter or spring (Sebunya and Saunders, 1983). Each of these factors can increase the level of stress incurred by swine. Optimal conditions to prevent an outbreak are ample space for housing the pigs, proper ventilation, limited mixing of the animals, and limited transportation (MacInnes and Rosendal, 1988; Ice et al., 1999; Dubreuil et al, 2000).

Epidemiology

The occurrence of pleuropneumonia in pigs is wide spread. Outbreaks have been reported in the U. S., Mexico, Canada, European countries, Japan, Australia, and South America (Sebunya and Saunders, 1983; Straw et al., 1999). Each of the *A. pleuropneumoniae* serotypes varies in their degree of virulence and by geographic location. Serotypes 1, 5, and 7 are prevalent in the United States. Serotype 2 is commonly found in Switzerland, Denmark, Japan, and Canada (Nielsen, 1988; Mittal et al., 1992). Serotype 8 is dominant in Europe, Mexico, and the United Kingdom (Dubreuil et al., 2000), and serotypes 1, 7, and 12 are prevalent in Australia (Prideaux et al., 1999). Isolates of serotypes 1, 3, 4, and 6 have been reported in England and serotypes 9 and 12 are dominant in France (Mittal et al., 1992). All serotypes of *A. pleuropneumoniae* can cause disease, but the degree of virulence is related to biotype, serotype, and strain. Overall, biotype 1 serotypes are more virulent than biotype 2 serotypes and within biotype 1 serotypes 1a, 1b, 5a, 5b, 9, and 10 seem to be more virulent (Haesebrouck et al., 2001). Serotype 1 appears to be the most virulent, while serotype 3 appears to be one of the least virulent. More virulent and less virulent strains however, may be found among different serotypes such as serotype 3 (Mittal et al., 1992). Serotypes 2, 4, 6, 7, 8, and 12 are considered less virulent and cause decreased mortality. Serotypes 1, 5, 9, and 11 are frequently involved in severe outbreaks with increased mortality (Donachie et al., 1995). Serotypes 2 and 9 have been isolated in European countries, but are less virulent and cause less mortality, but produce lung lesions similar to serotypes 1 and 5 (Moral et al., 1999).

Due to the prevalence, contagiousness, and severity of the disease, serotype identification is important to monitor the spread of disease, for development of serotype-specific vaccines, and for epidemiological studies. Serotyping however cannot provide detailed epidemiological information due to the limited number of serotypes present in a location (see below). Restriction endonuclease analysis (REA) has been used and proven useful to differentiate strains of identical *A. pleuropneumoniae* serotypes. REA was used to analyze *A. pleuropneumoniae* field isolates from southern Ontario. Except for serotypes 1 and 9, all serotypes had very distinguishable profiles. The only differences were serotypes 1 and 5 isolates, which revealed limited heterogeneity and serotype 7 isolates that showed a greater variation in the REA profile (MacInnes et al., 1990). REA was also used to analyze 17 isolates from New Zealand. Cleavage patterns allowed the 17 isolates to be differentiated from other *A. pleuropneumoniae* reference strains and grouped into 7 profiles based on chromosomal DNA analysis and plasmid profiling (Wards et al., 1998).

Ribotyping is another method that has been used for epidemiological studies. It was used in Denmark to study routes of transmission in a specific pathogen- free herd. The use of ribotyping allowed the differentiation of cases in which airborne transmission was indicated in 5 of 12 cases, and transmission by pig transporting vechicles was indicated in 6 of 9 cases (Fussing et al., 1998).

Clinical Signs

Pleuropneumonia can occur in pigs of all ages, but growing pigs 2-6 months of age are particularly susceptible. An outbreak of *A. pleuropneumoniae* can be present with varying degrees of clinical disease due to the immune status of the herd and the infectious serotype. Disease can range from peracute to acute to chronic (Byrd et al., 1992; Bosse et al., 2002). During the peracute stage, pigs quickly become ill develop a high fever, apathy, and anorexia. Vomiting and diarrhea may occur for a short period of time. The affected pigs lie on the ground with an increased respiratory rate and cyanosis becomes evident in the skin (Shope, 1964). In the terminal phase and before death, there is severe dyspnea, and a foamy, blood-tinged discharge may be present in the mouth and nostrils. Some pigs may also die abruptly without visible clinical signs (Sebunya and Saunders, 1983; Straw et al., 1995; Bosse et al., 2002). Peracute infection is more common in herds that have not had previous contact with *A. pleuropneumoniae* (Mittal et al., 1982).

Acute out breaks are characterized by hemorrhagic necrotizing pneumonia with increased morbidity and mortality. In the acute form pigs develop fever, areas of the skin may redden, animals are reluctant to eat or drink and are very lethargic. Respiratory distress with dyspnea and cough are evident. Failure of the circulatory system and cardiac failure is also present. There is an apparent loss of condition, which is visible within 24 hours of disease onset (Straw et al., 1999).

Pigs that survive an acute infection often develop chronic lung lesions and may become carriers (MacInnes and Rosendal, 1988). Chronically infected animals appear healthy, but harbor *A. pleuropneumoniae* in the upper respiratory tract and in the tonsils and nasal passages (MacInnes and Rosendal, 1988; Savoye et al., 2000). Economic losses are in large part due to chronically infected swine. In chronically infected swine, acute signs of disease have disappeared, there is little or no fever, and a slight intermittent cough may be present. There may be a reduction in appetite that contributes to decreased weight gain. Inefficient feed conversion, increased medication cost, and reduced market value are other characteristics of chronically infected animals (MacInnes and Rosendal, 1988; Chiers et al., 2002). These clinically inapparent infections are at times only evident at necropsy by the presence of unresolved lung lesions (Sebunya and Saunders, 1983). Subclinical infection is also commonly seen in chronically infected herds.

Pathology

The disease produced by *A. pleuropneumoniae* is characterized by hemorrhagic necrotizing pneumonia and fibrinous pleuritis. Swine that have been infected with *A. pleuropneumoniae* display gross pneumonic lesions in the respiratory tract upon necropsy. The pneumonia is usually bilateral involving the caudal lobes, but also occur on the cranial and median lobes (Shope, 1964; Dubreuil et al., 2000). Lung lesions from acutely affected swine are purplish red in color and interlobular septal thickening occurs as a result of inflammatory cell and fibrinous exudates and hemorrhage. The trachea, bronchi, and thoracic cavity contain blood tinged mucus exudate, and fibrinous pleurisy is evident (Shope, 1964; Didier et al., 1984; Straw et al., 1999). Many studies have

determined that the pulmonary lesions produced are a result of the *A. pleuropneumoniae* exotoxins. This was demonstrated when lesions similar to those seen in natural disease were produced by intratracheal innoculation of a sonicated supernatant of *A. pleuropneumoniae* (Bertram, 1988).

Early cellular changes that occur include polymorphonuclear infiltration, edema, and fibrinous exudate. Pleomorphic fibroblasts line the wall of the interlobular septa. In the later stages, macrophage infiltration is more evident and the necrotic areas are surrounded by degenerating leukocytes. Degenerating PMN's and pulmonary epithelial cells are found within alveoli. In the lung, degenerating fibrin and erythrocytes are found in dilated capillaries (Pattison et al., 1957; Bertram, 1988; Byrd et al., 1992).

In pigs that survive infection, lesions may shrink and the dark red color fades, but in chronic cases, different size nodules remain in the diaphragmatic lobes with overlying areas of fibrinous connective tissue that sequester the area of necrosis (Straw et al., 1999 Dubreuil et al., 2000; Bosse et al., 2002). *A. pleuropneumoniae* is primarily detected in neutrophils and macrophages at the edge of the tissue surrounding the necrosis (Cho and Chae, 2001a). Animals that are chronically infected harbor *A. pleuropneumoniae* in the tonsils or in the necrotic lesions. These swine frequently become asymptomatic carriers and can introduce *A. pleuropneumoniae* into previously non-infected herds (Bosse et al., 2002). Due to the limited number of bacteria that would be introduced by these carriers, shedding such bacteria may provide some herd immunity against the disease (Straw et al., 1999).

Virulence Factors

Capsule

There are many different factors that can contribute to the pathogenicity of *A. pleuropneumoniae*. Three of the most important and best characterized are the capsule, lipopolysaccharide, and exotoxins. The following is a summary of these virulence factors and other potential virulence factors.

The capsular polysaccharide is an important virulence factor of *A. pleuropneumoniae* and is responsible for serotype-specificity (Ward and Inzana, 1997). The chemical structure and composition of the capsular polysaccharide of all twelve serotypes has been determined (Perry et al., 1990). The capsules are negatively charged. In serotypes 2, 3, 6, 7, 8, 9 and 11 the negative charge is due to phosphate residues, in serotypes 5a, 5b and 10 the negative charge is due to phosphate diester bonds and in serotypes 1, 4 and 12, the negative charge is due to phosphate bonds (Perry et al., 1990; Dubreuil et al., 2000).

The capsular polysaccharide has been shown to be important for pathogenesis, and for diagnostic and epidemiological purposes (Inzana, 1991; Donachie et al., 1995). Certain serotypes of *A. pleuropneumoniae* are more virulent than others. These variations in virulence may be related to the structure or amount of capsule produced and the exotoxins made (Dubreuil et al., 2000). Electron microscopy has shown that more virulent strains of a given serotype have a larger and more adherent capsule, while less virulent strains have a smaller, more fragile capsule (Steffens et al., 1990; Inzana, 1991). Jensen and Bertram (1986) demonstrated this with strains of serotype 5: one strain had a

less adherent capsule and was shown to be less virulent than another strain of the same serotype containing a more adherent capsule and was more virulent. Jacques et al. (1988) also demonstrated the variation in capsule structure when the capsules of serotypes 1-10 were analyzed by electron microscopy and determined to have a capsule thickness of 80-90 nm to 210-230 nm depending on the serotype. This may partially explain differences in virulence among different serotypes.

Capsule protects the bacterium from host defenses such as phagocytosis and complement-mediated killing. Encapsulated strains of *A. pleuropneumoniae* are resistant to complement-mediated killing (Ward and Inzana, 1997). Non-encapsulated mutants of serotype 5 are effectively killed in the presence of serum but encapsulated strains are not (Ward and Inzana, 1994). In comparison to the serotype 5 mutant, a non-encapsulated mutant of serotype 1 was not killed in the presence of serum (Ward and Inzana, 1994; Rioux et al., 2000). Non-encapsulated mutants isolated following mutagenesis are able to induce protective immunity and did not cause clinical symptoms or lung lesions after intratracheal challenge (Inzana et al. 1991, Inzana et al., 1993).

The capsular polysaccharide of *Escherichia coli* has been studied the most intensely of all gram-negative bacteria. The genetic organization of the *A. pleuropneumoniae* capsular gene loci is similar to the organization of the group III capsule of *H. influenzae* type b, and *N. meningiditis* group B, and the type II capsules of some *E. coli* strains (Ward et al., 1998). *A. pleuropneumoniae* however, appears to produce a type III family capsule. Both capsule groups have a common segmental organization containing a serotype-specific region (region 2), which encodes proteins

involved in synthesis of capsule, flanked by conserved genes (regions 1 and 3) that encode proteins involved in polysaccharide export (Russo et al., 1998; Clarke et al., 1999).

Exotoxins

Most strains of *A. pleuropneumoniae* produce one or more hemolysins. Analysis of these hemolysins revealed them to be a pore-forming protein of the RTX (Repeat in ToXin)-toxin family (Donachie et al., 1995; Shaller et al., 1999). These toxins are found in a wide variety of Gram-negative bacteria such as *Bordetella pertussis, M. hemolytica,* and *E.coli*. (Prideaux et al., 1998). In *A. pleuropneumonaie* these toxins are called Apx-toxins and are identified as ApxI, ApxII, ApxIII (Frey, 1995) and ApxIV (Schaller et al. 1999, Schaller et al., 2001, Cho and Chae, 2001b). Apx toxins are believed to play an important role in the pathogenesis of *A. pleuropneumoniae*. It is likely that the degree of virulence associated with the different serotypes, is largely due to the exotoxins expressed (Frey, 1994). Each of these toxins varies in its hemolytic and cytotoxic activity. (Donachaie et al., 1995; Frey, 1995). Serotypes 1, 5, 9 and 11 are particularly virulent and produce ApxI, which is strongly hemolytic and cytotoxic (Frey et al., 1995).

Apx I toxin is strongly hemolytic and cytotoxic to phagocytic cells. It strongly binds calcium which regulates the expression of *apxI* (Frey, 1988). The binding of calcium is dependent on the number of glycine-rich nonapeptide repeats. An increased number of repeats produces increased binding of calcium (Donachie et al., 1995). ApxI has 13 glycine-rich nonapeptide repeats (Maier et al. 1996). The entire Apx I operon is present in serotypes 1, 5a, 5b, 9, 10, and 11 (Jansen et al., 1994; Frey et al., 1995).

However, serotypes that do not produce ApxI, (serotypes 2, 4, 6, 7, 8 and 12) do contain the *apx*IBD portion of the operon (Jansen et al., 1993; Jansen et al., 1994). The Apx I protein has a molecular mass of about 105 kDa. The nucleotide sequence of the Apx I operon has been determined and is made up of four genes. These genes are arranged in the order *apxIC*, *apxIA*, *apxIB* and *apxID* respectively. The *apxIC* gene was determined to be the pre-toxin activator gene and *apxIA* was determined to be the pre-toxin structural gene, which has strong similarity (60%) to the *E. coli hlyA* gene. The *apxIB* and *apxID* genes encode for proteins required for transport of the activated toxin (Prideaux et al., 1999).

ApxII is weakly hemolytic and moderately cytotoxic and is secreted by all serotypes except serotype 10. The ApxII protein has a molecular mass of 103-105kDa and contains only 8 glycine-rich nonapeptide repeats (Frey and Nicolet, 1988; Maier et al., 1996). ApxII requires Ca^{2+} for expression (Frey, 1988). ApxII is encoded for by an operon containing only the *apxIIC* and *apxIIA* genes, which are expressed as a single RNA transcript. Secretion of ApxII is dependent on the activity of the *apxIB* and *apxID* genes, which are present in all serotypes except serotype 3. Due to the absence of the *apxIB* and *apxID* genes required for secretion in serotype 3, the ApxII toxin is mainly cytotoplasmic (Frey, 1995). ApxIIA has 67% similarity to the *M. haemolytica* LktA leukotoxin and has 47% similarity to the *E. coli hlyA* gene (Chang et al., 1989; Frey, 1995; Prideaux et al., 1998).

ApxIII is non-hemolytic, and strongly cytotoxic for alveolar macrophages and neutrophils. It is produced and secreted by serotypes 2, 3, 4, 6 and 8. The ApxIII protein

has a predicted molecular mass of 120kDa and contains 13 glycine-rich nonapeptide repeats (Maier et al., 1996). The ApxIII toxin is encoded for by an operon similar to that of ApxI. The operon has the *apxIIIC*, *apxIIIA*, *apxIIIB* and *apxIIID* genes, which are expressed from a single promoter. The ApxIII toxin has 54% to the HlyA hemolysin of *E. coli* (Maier et al., 1996).

A fourth type of pore-forming RTX protein was recently identified and designated ApxIVA. The *apxIV* gene is expressed *in-vivo* and cannot be detected in cultures grown under various in-vitro conditions. All twelve *A. pleuropneumoniae* serotypes are determined to contain the *apxIV* gene (Cho and Chae, 2001a; Cho and Chae, 2001b; Schaller et al., 2001). The gene encoding ApxIV was cloned and characterized from *A. pleuropneumoniae* serotype 1 strain 4074 and from *A. pleuropneumoniae* serotype 3 field strain HV114. The *apxIV* gene is preceded by ORF1, which seems to be involved in activation of ApxIV. In comparison to the other Apx toxins, ApxIV resembles ApxII by showing weak hemolytic activity and also producing the Christie-Atkins-Munch-Petersen (CAMP) reaction. Structurally, ApxIV has characteristics of RTX proteins, such as the repeated glycine-rich nonapeptides at the C-terminal end known to bind calcium, N-terminal hydrophobic domains, and potential acylation sites (Cho and Chae, 2001b). Biotype 2 strains do not express all toxins made by corresponding biotype 1 strains. None of the biotype 2 serotypes produce ApxIII, but they do contain the *apxI* and *apxII* genes and produce the ApxI and ApxII toxins (Donachie et al., 1995).

The role of Apx toxins in virulence has been demonstrated by the ability of culture supernatant to cause clinical disease and produce typical pneumonic lesions in swine.

Swine innoculated with culture supernatant depleted of Apx toxins were clinically normal and showed few symptoms (Kamp et al., 1997). It has also been demonstrated that Apx toxins can potentially be used as vaccine candidates. Prideaux et al. (1998) reported the construction of a serotype 7 mutant that lacked the *apxIA* and *apxIC* genes and was attenuated in a mouse model. Prideaux also reported that expression of the ApxA structural toxin in non-activated form on a vector in the attenuated mutant, produced Apx-specific protective antibodies against the homologous serotype 7 strain and a heterologous serotype 1 strain.

Lipopolysaccharide

The lipopolysaccharide (LPS) is a major component of the outer membrane of *A. pleuropneumoniae* and has been shown to induce tissue damage. The lesions produced by purified LPS, which are neither hemorrhagic nor necrotic, are different from typical lesions of pleuropneumonia. LPS most likely acts in conjunction with the Apx toxins to amplify the virulence and severity of the Apx toxins of *A. pleuropneumoniae* (Haesebrouck et al., 2001).

LPS has also been determined to be important in the adherence of many gram-negative bacteria to host cells. LPS is the major component involved in adherence of *A. pleuropneumoniae* to porcine respiratory cells and to cultured porcine tracheal rings (Paradis et al., 1994; Rioux et al., 2000). The LPS is composed of three distinct regions: lipid A, which contains a glucosamine backbone and fatty acids, is responsible for the toxic properties of LPS; (Hammond et al., 1984) the core region, which is composed of an oligosacccharide containing 2-keto- 3-deoxy-*D-manno*-2-octulosonic acid, and the O-

antigen, which is a polysaccharide chain consisting of repeating glycose units. In *A. pleuropneumoniae*, LPS can be have long O-side chains, intermediate length O-side chains or short O-side chains (Perry et al., 1990; Inzana, 1991; Paradis et al., 1999). Many of the *A. pleuropneumoniae* serotypes have a structurally unique LPS, and differ in the structure of the O-side chains, but contain common epitopes. These common epitopes are responsible for cross-reactions between serotypes 3, 6, and 8, 4 and 7, and 1, 9, and 11 (Nielsen et al., 1996; Paradis et al., 1999). Serotypes 2, 4, and 7 have long O-side chains, serotypes 1 and 5 have intermediate length side chains, and serotypes 3, 6, and 8 have short O-side chains. Short O-side chains have also been found in serotype 5. These epitopes may be serotype-specific or they may cross-react with-in the species or between species (Fenwick et al, 1986).

Adherence is the initial event that allows colonization and enables the bacteria to exert its pathogenic properties and cause disease (Paradis et al., 1999; Bosse et al., 2002). Bacterial adhesins and host receptors mediate adherence of *A. pleuropneumoniae* to epithelial cells. Receptors however, are different and bind different regions of the LPS (Prideaux et al., 1999; Paradis et al., 1999). Previous studies suggested that the high molecular mass polysaccharide portion of the LPS was involved in adherence. Recently it was discovered that LPS mutants lacking high molecular mass polysaccharide, adhered to frozen tracheal sections in higher numbers than the wild type. Due to these results, the role of high-molecular-mass- polysaccharide in adherence may need to be re-examined (Bosse et al., 2002). It has also been shown that purified LPS with long O-side chains can inhibit adherence of *A. pleuropneumoniae* to frozen tracheal sections, which further

indicates a role of O-side chains in adherence (Paradis et al., 1999). Surface exposure of the LPS seems to play an important role in adherence. LPS was exposed on the surface of *A. pleuropneumoniae* and other encapsulated bacteria such as *Klebsiella pneumoniae* and this is essential for adhesion to occur. Capsule can block the interaction between the LPS and respiratory tract mucus depending on the amount of capsule produced (Donachie et al., 1995). The type of LPS present also affects adhesion. LPS with long O-side chains was shown to bind more effectively to cultured porcine tracheal rings than LPS with intermediate length O-side chians (Donachie et al., 1995; Bosse et al., 2002).

Outer Membrane Proteins

Outer membrane proteins (OMP) have been implicated as a virulence factor of *A. pleuropneumoniae*. The outer membrane protein profiles differ for most *A. pleuropneumoniae* serotypes, but all serotypes contain some common outer membrane proteins. The 14 kDa Pal A, peptidoglycan-associated lipoprotein, a 29/41 kDa heat modifiable protein, the 48 kDa Aop A protein (Cruz et al., 1996), and a major protein that varies from 32-42 kDa depending on serotype, are all common outer membrane proteins (Maier et al., 1996). The Pal A and 32 and 42 kDa proteins are immunologically dominant. A 40 kDa minor OMP (OmlA) was identified and determined to be present in all 12 *A. pleuropneumoniae* serotypes (Osaki et al., 1997). Western blot analysis showed that convalescent sera from *A. pleuropneumoniae* infected pigs contained antibodies against these outer membrane proteins and other common outer membrane proteins (Maier et a1., 1996). The use of outer membrane proteins for vaccines or as part of

vaccine components has been evaluated. A recombinant OmlA protein from *A. pleuropneumoniae* serotype1 was used to vaccinate swine and did lower mortality, but lung lesions and *A. pleuropneumoniae* were still present (Gerlach et al., 1993). The *omlA* from serotype 5 was also cloned and sequenced. Vaccination with this recombinant protein, protected swine from death against challenge with serotype 5 (Belanger et al., 1995). Serotypes 1-5 and 7 produce OMP of 105 and 76 kDa under iron-restricted conditions and convalescing sera react with these proteins (Inzana, 1991). The use of the transferrin binding lipoprotein (TbpB) was effective in producing a strong immune response in swine and reducing mortality and clinical signs of disease after challenge (Fuller et al., 2000).

Superoxide Dismutase

Superoxide dismutase (SOD) is a potential virulence factor because of its ability to aid bacterial survival during an infection. Bacterial pathogens are exposed to increased levels of superoxide radicals during infection. The SOD's are involved in cellular defense against oxidative damage by dismutating cytotoxic radicals to hydrogen peroxide and oxygen (Chen et al., 2001, Kroll et al., 1995). The inflammatory response to an *A. pleuropneumoniae* infection predominantly involves neutrophils. Neutrophils generate oxygen radicals that can cause severe tissue damage when produced in excess (Cho and Chae, 2001a). The SOD's prevent the accumulation of cytotoxic free oxygen radicals thereby protecting the bacteria from death. The *sodC* gene, which encodes the copper- and zinc- cofactored SOD, has been cloned and sequenced in *A. pleuropneumoniae* serotype 1. A *sodC* mutant of *A. pleuropneumoniae* serotype 1 was constructed and did

show increased sensitivity to the superoxide present in vitro, but remained fully virulent and was able to cause acute pleuropneumonia in experimentally infected pigs (Sheehan et al., 2000; Bosse et al. 2002).

Iron Binding Proteins

Iron is a nutritional requirement for pathogenic bacteria, but it is usually complexed with other molecules such as transferrin, lactoferrin and hemoglobin. Many microorganisms can produce or use some type of iron binding compound by secretion of siderophores that allow utilization of the complexed iron. *A. pleuropneumoniae* however, does not secrete siderophores but produces transferrin receptor binding proteins under iron limiting conditions. *A. pleuropneumoniae* can use porcine transferrin, hemoglobin, and some porphyrin compounds as iron sources, but cannot use bovine, human, or other transferrins (Diarra et al., 1996; Bosse et al., 2002). *A. pleuropneumoniae* produces two transferrin binding receptor proteins under iron restricted growth conditions. These transferrin binding receptor proteins are TbpA and TbpB. TbpA has a molecular mass of 100kDa and forms a transmembrane channel for iron transport across the outer membrane. TbpB has a molecular mass of 60 kDa, is a lipoprotein anchored to the outer membrane and has also been shown to bind hemin. Both transferrin binding receptor-binding proteins bind the C-lobe of porcine transferrin and the interaction of the two proteins is needed for optimal utilization of transferrin. The *tbpA* and *tbpB* genes are linked with the *exbD* genes. ExbB and ExbD form an inner membrane protein complex associated with TonB and provides energy to high-affinity receptors allowing transport of iron across the outer membrane (Bosse et al., 2002). Mutants of *A. pleuropneumoniae*

serotype 7 containing a deletion in the *tbpA* or *tbpB* genes did not cause clinical disease, and no immune response to the serotype 7 mutants was detected 21 days after infection (Baltes et al., 2002). Tbp when used to immunize pigs, was able to confer limited protection against challenge with the homologous strain (Haesebrouck et al., 2001).

Fimbriae

Bacterial colonization requires attachment of the bacteria to host cells. In addition to LPS, fimbriae may be involved in the attachment process thereby facilitating the initial steps leading to infection. Type 4 fimbriae are found in many gram-negative bacteria such as *Moraxella bovis*, *Neisseria gonorrhoeae*, *N. meningitidis*, and *Vibrio cholerae* (Zhang et al., 2000). Since it has been established that *A. pleuropneumoniae* attaches to epithelial cells in the respiratory tract, the presence of fimbriae would be beneficial to enhance the attachment process. The presence of fimbriae has been observed by transmission electron microscopy in *A. pleuropneumoniae* field isolates (Inzana 1991; Utrera and Pijoan, 1991; Baltes et al., 2002). Fimbrial size was determined to be 0.5-2.0 nm in diameter and 60-450 nm in length (Utrera and Pijoan, 1991; Straw et al., 1995). Fimbriae determined to be type 4 have also been purified from serotypes 1, 2, 7, and 12 (Bosse et al., 2002). Fimbriae production in *A. pleuropneumoniae* seems to be phase variable. Utrera and Pijoan (1991) reported that fimbriae were detected in only half of the field isolates grown on blood agar and were not detected after the second passage. Fimbriae were also non-detectable when grown on media with out blood or on BHI-NAD. Due to the apparent instability of fimbriae production, it is suspected that

fimbriae may be expressed under in-vivo conditions only (Inzana 1991, Bosse et al., 2002).

Proteases

A. pleuropneumoniae secrete proteases, which may contribute to their pathogenesis during an infection. These proteases degrade porcine gelatin, IgA, IgG, and hemoglobin. Secreted proteases from serotype 1 have been reported to degrade porcine, bovine and human hemoglobin (Donachie et al., 1995). The cleavage of hemoglobin may be a method used by the pathogen to acquire iron (Inzana, 1991; Haesebrouck et al., 2001). Negrete-Abascal et al. (2000) reported the purification and characterization of a > 200kDa metalloprotease, which is an antigen common to all 12 serotypes. This metalloprotease was also able to degrade gelatin. Negrete-Abascal et al., (2000) reported the discovery of a 24 kDa zinc metalloprotease present in all 12 serotyes, and which showed in-vitro cleavage of actin (Garcia-Cuellar et al., 2000). It has been reported that some gram-negative bacteria release vesicles that contain outer membrane-associated toxins and enzymes (Myhrvold et al., 1992). They were first observed in *A. pleuropneumoniae* and have been characterized in serotype 1. The membrane vesicles from serotype 1 are determined to contain proteases, and Apx toxins, which when secreted into host tissues, could contribute to the damage of host defense molecules and the production of favorable conditions to allow growth of *A. pleuropneumoniae* (Negrete-Abascal et al., 2000).

Prevention and Control

Several different methods can be used for the prevention and control of pleuropneumonia. Antibiotic treatment, vaccination, and improved management and husbandry practices are a few techniques that can be used alone or in combination. Antibiotic therapy on a regular or intermittent basis can and has been used for the prevention of disease. However, on a long-term basis, antibiotic therapy would promote antibiotic resistance (Chiers et al., 2002). In herds that are free from disease and infection, isolation of the herd is essential to maintain the absence of infection. Any new animals brought into the herd should be purchased from known disease-free or specific-pathogen free herds (MacInnes and Rosendal, 1988; Straw et al., 1999). The new animals should be quarantined before introduction into the herd and it is also worthwhile to test them for infection or specific antibody prior to introduction. An all-in all-out policy should also be implemented (Straw et al., 1999). A study, which compared the use of all-in-all-out management to continuous flow management, demonstrated that all-in-all-out management was better overall for health and growth of pigs and could reduce the prevalence and severity of pneumonia in pigs of all ages (Ice et al., 1999).

Many herds may already be subclinically infected due to exposure to a minimal number of organisms or due to infection with a less virulent serotype such as serotype 3. Some of these subclinically infected pigs may be culture negative but seropositive, which shows that *A. pleuropneumoniae* can colonize the upper respiratory tract without seroconversion occurring (Savoye et al., 2000). Some farmers may allow the subclinically infected animals to remain, due to studies that show recovery from a natural

or experimentally induced infection produces immunity against homologous and heterologous serotypes (Nielsen, 1979). Subclinically infected animals however, are carriers and could expose non-infected animals, which may increase the threat of an acute outbreak (Dubreuil et al., 2000).

Once a herd has become infected, improvement of management conditions and housing conditions is important to minimize stress factors and control environmental factors. Crowding and sudden climatic changes are a few stress factors that can influence the likelihood of an outbreak (MacInnes and Rosendal, 1988). Factors such as temperature, ventilation and the use of partitions between pens may minimize the spread and severity of disease, specifically in fattening units. Antibiotic therapy can be used to treat pleuropneumonia and has been shown to reduce mortality and improve average weight gain. However, effectiveness of antibiotic therapy is usually limited to the early stages of infection, and the infection may not be completely eliminated (Straw et al., 1999; Chiers et al., 2002). Due to the rapid onset and severity of disease, however, the loss of swine may occur before antibiotics have had time to become effective (Belanger et al., 1995). Antibiotic treatment is not effective in chronically infected animals and animals that have been treated can still carry the disease (MacInnes and Rosendal, 1988). Development of antibiotic resistance by *A. pleuropneumoniae* serotypes, such as 1, 3, 5, and 7, has been shown to be plasmid mediated and may also affect the decision to treat an infected herd (Chang et al., 2002).

Immunization of herds to prevent or control infection with *A. pleuropneumoniae* has been used and does reduce mortality, but does not prevent initial infection or the

development of carrier animals (Goethe et al., 2001; Chiers et al., 2002). The use of vaccines has proven difficult due to the occurrence of 2 biotypes and more than 14 serotypes of *A. pleuropneumoniae*. The cross-protection against other serotypes observed with vaccines has not been adequate (Belanger et al., 1995; Straw et al., 1999). Killed whole-cell and subunit vaccines have been developed for protection against *A. pleuropneumoniae*. Vaccination with killed whole cells from one serotype does not protect against other serotypes. Current vaccines are killed-whole cell or live attenuated. Live attenuated vaccines do reduce mortality, but do not prevent morbidity and swine may still experience chronic disease (Inzana et al., 1993; Maier et al., 1996; Fuller et al., 2000). Two of the subunit vaccines developed were recombinant proteins of a truncated RTX toxin and a regulatory transferrin iron binding protein. Immunization with these vaccines provided protection from death against challenge with serotype 7, but could not prevent lung damage or survival of the bacteria, and did not provide cross-protection against *A. pleuropneumoniae* serotype 1 (Gerlach et al., 1993; Goethe et al., 2001). The expression of a cloned recombinant form of an outer membrane lipoprotein (OmlA) was also used and vaccinated animals were protected from death (Belanger et al., 1995).

Identification and Serology

A. pleuropneumoniae can be isolated from tissues or secretions by inoculation to chocolate agar or by cross-streaking onto 5% sheep blood agar and then inoculating the plate with a single streak of *Staphylococcus aureus*. *A. pleuropneumoniae* colonies will appear adjacent to the *S. aureus* streak due to the latter's production of NAD (Bosse et al., 2002). Biochemical identification of *A. pleuropneumoniae,* can be carried out by demonstrating urease activity, by the ability to ferment xylose, ribose, and mannitol and by enhanced β-hemolysis adjacent to *S. aureus* (Sebunya and Saunders, 1983; Straw et al., 1999).

There are 12 serotypes of *A. pleuropneumoniae* each of which vary in virulence and by geographic location. Serotypes 1, 5, and 7 are most common in the United States whereas other serotypes such as 2 and 9 are more prevalent in European countries. Serotype classification of *A. pleuropneumoniae* is based on the capsular polysaccharide antigen (Inzana and Mathison, 1987; Lo et al., 1998). Serotyping is important in order to control the spread of the disease, for epidemiological purposes, to confirm the identity of serotypes that are known to be prevalent in a geographic location, and to identify new serotypes that may become prevalent in a geographic location (Mittal et al., 1987; Nielsen et al., 1996). Despite advancement in serotyping procedures, cross-reactions between serotypes still occur. Specifically, cross-reactions between serotypes 1, 9, and 11, serotypes 3, 6, and 8 and serotypes 4 and 7 are the most common (Nielsen et al., 1996; Chevallier et al., 1998; Dubreuil et al, 2000). These cross-reactions are a result of shared

species-specific antigens such as the LPS O-side chain or membrane proteins (Perry et al., 1990).

Serological Assays

Complement Fixation Test

The complement fixation test (CFT) was one of the earliest tests used to serodiagnose *A. pleuropneumoniae*. The CFT is an assay, which uses complement to detect antigen–antibody complexes. This summary of the CFT is described by Mittal et al. (1984). Antigens are prepared by suspension of bacterial growth in formalinized saline or the suspension could be extracted with phenol as reported by Gunnarsson (1978); This is considered whole cell antigen. Checkerboard titration of both antigen types reveals the optimal dilution for the maximum titer. The CF test is carried out in microtiter plates. Test serum is added to each well followed by antigen and then guinea pig complement. Plates are incubated overnight and then sheep red blood cells are added to each well. Plates are incubated for one hour and then read by placing a concave mirror under the microplate. A positive result is determined by the presence of unlysed RBC's on the bottom of the well, forming a button. A negative reaction is determined by the absence of unlysed RBC's. The reciprocal of the highest dilution of serum having approximately 50% hemolysis is determined as the end-point titer (Mittal et al., 1984).

The CFT has been used for many years and was the accepted method in Switzerland, Denmark, Canada, and the United States for the detection of pigs infected with *A. pleuropneumoniae*. The assay does not accurately detect recently infected or

chronically infected herds, due to a low titer or the absence of a titer. Results from a CFT should be interpreted on a herd basis. The CFT is laborious, time consuming, and expensive. Therefore, it is not routinely used today except in Denmark, Switzerland, and a few labs in the United States (Nielsen, 1988; Dubreuil et al., 2000).

Indirect Hemagglutination Test

The indirect hemagglutination (IHA) test is another test that detects the presence of antibodies in serum. Nielsen (1974) was the first to use the IHA test for the diagnosis of *A. pleuropneumoniae* in swine. IHA tests have been used for the identification of various *Pasturella* species. The following is the procedure for the IHA test as described by Mittal et al. (1983b). Antigen is prepared by suspending bacterial cells in a saline solution supplemented with IsoVitaleX. The solution is centrifuged and the supernatant (saline extract) is collected. The saline extract is incubated with the SRBC's so that absorption of the antigen by the SRBC's will occur. The sensitized SRBC's are ready to be used in the IHA test. Sera is prepared by heating at 56^0 C for 30 minutes and then adsorbing the serum with unsensitized SRBC's to remove non-specific antibodies. The sera is diluted and pipetted into the wells of a microtiter plate. Sensitized SRBC's are added to the wells of the microtiter plate and the wells are mixed. The plates are incubated and a positive reaction is determined by visualization of flat sediment at the bottom of the well, while a negative reaction is seen as a smooth dot at the bottom of the well. The titer of the IHA is the reciprocal of the highest dilution of sera yielding a positive result.

The IHA test has been used to differentiate cross-reacting strains of *A. pleuropneumoniae* such as serotypes 3, 6, and 8, 4 and 7, and 1and 9. The use of IHA to differentiate serotypes 9 and 11, however, has been problematic (Blackwell et al., 1999). Nielsen showed that serotypes 6 and 8 share antigenic determinants, which are polysaccharide in nature and part of the capsular substance (Nielsen and O'Connor, 1984). An antigenic relationship was also seen with nine biotype 1 Danish isolates and *A. pleuropneumoniae* serotypes 2 and 7. The shared antigens were determined to be polysaccharide in nature and probably LPS (Nielsen et al., 1996). These cross-reactions are thought to be a result of heat labile and heat stable antigens, primarily IgG type antibodies. Cross-reactions were decreased when tests such as the 2-mercaptoethanol tube agglutination test and 2-ME IHA were used (Mittal et al., 1988). Nielsen also reported subtypes of *A. pleuropneumoniae* serotype 5, 5a and 5b, based on antigenic differences in capsular antigens (Nielsen, 1986).

Enzyme Linked Immunosorbant Assay (ELISA)

The ELISA is another assay used to detect antibodies to specific bacterial components such as capsule, LPS, toxin, etc. Compared to the complement fixation test, the ELISA was determined to be more sensitive and specific for antibody detection (Inzana and Fenwick, 2001). The following is a basic procedure described by Klausen et al. (2001). *A. pleuropneumoniae* cells are grown and harvested, and the LPS antigen to be used is extracted, diluted out by checkerboard titration to determine optimal concentration and then used to coat the wells of a microtiter plate. The plate is incubated at 4^0 C overnight and then

non-specific sites are blocked with blocking solution. Test serum that has been diluted is then added to the wells and the plate is incubated. Secondary antibody is added, which can be rabbit anti-swine or goat anti-swine IgG conjugated to horseradish peroxidase. The microtiter plate is washed and the substrate is added, which when bound to the secondary antibody, produces a colormetric reaction. The reaction is stopped in 15-20 minutes by the addition of 0.5M H_2SO_4 and the absorbance determined spectrophotometrically using a microplate reader.

The sensitivity and specificity of an ELISA can be affected by the quality of antigen preparation used. Nicolet et al. (1981) reported that the use of EDTA-treated antigen gave more favorable results than SDS-treated, phenol-extracted, or sonicated antigen in an ELISA, which was more sensitive and detected more positive reactions in an infected herd than did the CF test. The antigens used in an ELISA can vary and may be species-specific or serotype-specific. The *A. pleuropneumoniae* ApxII protein toxin was used as antigen in an ELISA, for routine detection of antibodies to *A. pleuropneumoniae*. The ELISA detected 243 seropositive animals out of 400 compared to the CF test, which only detected 170. This ELISA was used as a species-specific test (Lenier et al., 1999). Nielsen et al. (2000) used Apx I, Apx II and Apx III toxins as antigens in an ELISA to determine if a diagnostic assay using antibodies to the Apx toxins would be valuable. Due to the sensitivity observed from the ELISA and because the toxins are present in all serotypes, this type of ELISA may be used for initial screening. Inzana and Mathison (1987) determined that the capsular polysaccharide is a more appropriate and serotype-specific antigen to use in an ELISA for maximized sensitivity and specificity. Inzana and Fenwick

described an ELISA in which biotin was conjugated to capsular polysaccharide and the complex was bound to Streptavidin-coated plates. This CP-biotin-streptavidin ELISA proved to be very specific and its sensitivity approached that of radioimmunoassay (Inzana and Fenwick, 2001).

Blocking ELISA's have also been used to detect antibodies to specific serotypes of *A. pleuropneumoniae*. Nielsen et al. (1993) developed a blocking ELISA to detect antibodies to *A. pleuropneumoniae* serotypes 2 and 8 and Klausen et al. (2001) developed a blocking ELISA for the detection of antibodies to serotype 6. Both assays are determined to be more sensitive and specific than the CF test. However, cross-reactions did occur with serotypes 3 and 8 in the *A. pleuropneumoniae* 6 blocking ELISA (Nielsen et. al., 1991; Klausen et al., 2001).

Serotyping

Whole Cell Agglutination Tests

Various agglutination tests such as tube agglutination (TA), 2-mercaptoethanol tube agglutination (2-ME TA), and slide agglutination (SA) have been used for the detection and serotyping of A. *pleuropneumoniae* isolates. These tests are rapid, simple and similar to perform. Antigens for the agglutination tests are prepared from formalinized-saline bacterial suspensions that have been heat-treated. This is referred to as the whole–cell antigen suspension (Mittal et al., 1987). Antiserum for the agglutination tests is collected from rabbits, which have been immunized with formalinized whole-cell suspensions of *A. pleuropneumoniae* antigens. The antiserum is

serially diluted in saline solution for the tube or slide agglutination tests and in saline solution containing 0.1M 2-Mercaptoethanol for the 2-Mercaptoethanol tube agglutination test. An equal volume of antigen is added to dilute antiserum and the two are mixed. A positive reaction in the slide agglutination test can be seen immediately and the reaction is graded as 1- 4+ depending on the rapidness and size of the clumps formed. The tube agglutination tests require incubation before a positive reaction can be determined. The titer is determined to be the highest serum dilution that produces evident agglutination.

Comparison of the TA and 2-ME TA tests showed that when using a formalinized saline solution, the TA test was not suitable for serodiagnosis due to non-specific reactions that were frequently observed (Dubreuil et al., 2000). These non-specific reactions were observed when a non-infected herd, which tested negative by the complement fixation test tested positive by the TA test. These results may have been due to the detection of agglutinins to other microorganisms (Mittal et al., 1984). The 2-ME TA test, which has been a standard test for Brucellosis was evaluated for its ability to detect seroreactive pigs. IgM type antibodies are at times involved in cross-reactions and by inactivating them in the 2ME-TA test it shows that IgG antibodies are responsible for the agglutination reaction thereby increasing the serotype-specificity of the assay. The 2-ME TA test was shown to be more specific than the tube agglutination test and more sensitive than the CF test, specifically when detecting early infections. However, the 2-ME TA was less sensitive in detecting chronic infections (Dubreuil et al., 2000). The

2ME-TA test was also shown to be more useful than the coagglutination test in determining strains that share major serotype-specific antigens (Mittal et al., 1987).

The rapid slide agglutination test was commonly used for routine serotyping because of its simplicity. The assay is based on whole cell agglutination and requires encapsulated cells (Mittal et al., 1987; Mittal et al., 1992). The ability of the whole cell suspension to agglutinate with antisera varied between strains. This was seemingly due to complete exposure of serotype-specific antigens in some strains, and complete to partial exposure of antigens in other strains. Heating seemed to expose the serotype-specific antigens needed for the agglutination test, however it also may have exposed some common antigens, which resulted in the occurrence of cross reactions between serotypes (Mittal et al., 1987). Despite the fact that the slide agglutination test seemed rapid and easy (Mittal et al., 1992) it did require that the antigen preparation be carried out after the bacteria were isolated and were cloned by standard laboratory porcedures (Rosendal et al., 1981; Mittal et al., 1992). Even then, some isolates remained untypeable. *A. pleuropneumoniae* can undergo dissociation from an encapsulated to non-encapsulated form and the non-encapsulated version cannot be typed by whole cell agglutination assays (Rosendal et al., 1981). In addition, strains that were either polyagglutinating or autoagglutinating could not be typed by whole cell agglutination tests (Mittal et al., 1987).

Coagglutination Test

The Coagglutination (Co-A) test was developed to type non-agglutinating, auto-agglutinating and poly-agglutinating strains, which could not be typed by agglutination

assays (Mittal et al., 1987; Mittal et al., 1992). This assay is based on the interaction between antigens and specific IgG fixed on *S. aureus* protein A. One drop of the Co-A reagent is mixed on a glass slide with an equal volume of bacterial suspension. The mixture is stirred and rotated and examined against a dark background (Mittal et al., 1983a). The reaction is determined to be negative if macroscopic agglutination does not occur with in 2 minutes (Bouh and Mittal, 1999).

Some isolates, which were untypeable by agglutination tests, remained untypeable by the Co-A test when whole cell suspensions of antigen were used, until the suspension was heated. Isolates were then easily typed. Difficulty in typing the isolates may have been due to hidden serotype-specific epitopes that became exposed when heated (Mittal et al., 1987; Mittal et al., 1992). The Co-A test however, could not distinguish serotypes 3, 6, and 8, 4 and 7, and 1 and 9 until adsorbed sera was obtained (MacInnes and Rosendal, 1988). Final identification of the serotypes could be achieved using the agar gel immunodiffusion test or indirect hemagglutination test. A comparison of the Co-A test and the ring precipitation test determined the Co-A test to be more sensitive and specific in detecting serotype-specific antigens in bacterial suspensions. Despite this, there was still a high correlation between results obtained from the Co-A and ring precipitation tests (Mittal et al., 1983).

Ring Precipitation Test

The ring precipitation test is another quick and simple test used for the serotype-specific identification of *A. pleuropneumoniae*. The following method was described by Mittal et al. (1982). Test antigens are prepared by harvesting mucoid colonies from agar

plates and suspending them in a formalinized-saline solution. The suspension is centrifuged and a clear supernatant is obtained and used as antigen. Antiserum to specified *A. pleuropneumoniae* serotypes is prepared in rabbits by immunization with whole cells of the reference strain. The ring precipitation test is performed by aspirating sera into a Pasteur pipette, inverting the pipette, and sealing the tip. The serum is overlaid with test antigen and a positive reaction is visible by the formation of a clear ring of precipitate at the interface, which indicates the binding of antigen and antibody (Mittal et al., 1982). This assay was developed to aid in typing mucoid, non-mucoid, and autoagglutinating strains that could not be typed by agglutination assays (Mittal et al., 1983; Mittal et al., 1992).

Agar Gel Immunodiffusion Test

The Agar Gel Immunodiffusion Test (ID) is an assay that has been used by Nicolet to identify atypical or non-typeable strains of *A. pleuropneumoniae* (Nicolet, 1988). Antigens used in this assay are harvested from mucoid colonies of *A. pleuropneumoniae* and suspended in a saline solution containing merthiolate. Antiserum is collected from rabbits immunized with the antigen suspension. A 1% agar medium is used to perform the immunodiffusion test, which contains 4 mm wells placed 6 mm apart from each other and from a central well. Antigen is placed in the central well and antibody is placed in the surrounding wells. After the antigen and antibody has diffused into the agar, a positive result is indicated by the formation of a precipitin line (Nielsen and O'Connor, 1984).

The ID Test is an assay that has been used to differentiate isolates which showed cross-reactions, such as serotypes 3, 6, and 8, 1 and 9, and 9 and 11. This assay is also used for quantitation of serotype and group specific antigens (Mittal et al., 1992). This assay was used to type 8 strains of *A. pleuropneumoniae* biotype 2 that were isolated from Danish herds. The ID test determined that the strains possessed type-specific antigenic determinants, which were of LPS and capsular origin as well as species-specific antigens. All 8 strains were antigenically homogeneous and distinct from known serotypes of biotype 1 and 2 (Nielsen et al., 1997). Despite the fact that the ID test has been used to identify serotype-specific capsular antigens of *A. pleuropneumoniae*, it is a time consuming assay, it is not readily available in every lab, and there is difficulty in interpreting positive results (Nicolet, 1988).

Indirect Fluorescent Antibody Test

The indirect fluorescent antibody test was thought to be able to replace the agglutination and immunodiffusion tests. This test can be performed quickly and easily due to the fact that culture is not needed. Saline suspensions of antigen from each serotype are prepared and used to immunize rabbits. Antiserum is collected for use when antibodies to the homologous antigen are present in sera. Smears of culture or lung tissue are fixed on slides and a drop of diluted serum applied. After incubation, a drop of goat-anti rabbit serum conjugated with fluorescein-iso-thiocyanate is applied to the slide. Fluorescence is detected by visualization under a microscope at 1,000 x magnification. The IFAT was able to serotype 63 of 65 isolates and in comparison with the ID test, 23 of the 63 isolates tested

gave the same results. The IFAT also detected *A. pleuropneumoniae* in impression smears from 12 lungs and culturing of the lungs verified these results (Rosendal et al., 1981).

Latex Agglutination

The latex agglutination test is a very sensitive and specific antigen test and it is the quickest and simplest of all the antigen detection assays. Limited reagents are needed for this assay and it can be used in the field or in a lab setting. Inzana (1995) describes the following method for latex agglutination. The capsular polysaccharide from A. *pleuropneumoniae* is purified from culture supernatant and ultracentrifuged to remove the endotoxin. Hyperimmune rabbit serum is obtained by injecting rabbits with formalinized whole cells in adjuvent and then whole cells, until the antibody titer against capsule reaches >1:12,500, as determined by an ELISA. Hyperimmune serum against each serotype is adsorbed with a non-encapsulated mutant of the same serotype. IgG from the adsorbed serum is isolated by protein A affinity chromatography. The optimum amount of affinity purified IgG is covalently coupled to carboxylate latex particles. To perform the latex agglutination test, ten microliters of the sensitized latex particles (SLP's) are mixed with twenty-five microliters of sample on a dark agglutination slide. Results are scored 4+ (rapid agglutination within 10 seconds and ring formation), 3+ (agglutination with 75% of the SLP's and some ring formation), 2+ (agglutination with greater than 50% of the SLP's and no ring formation), 1+ (less than 25% agglutination) the results are questionable, and 0 (no visible agglutination) (Inzana, 1995).

Polymerase Chain Reaction

The polymerase chain reaction or PCR is a DNA based assay that has become a popular new tool for the detection and serotyping of *A. pleuropneumoniae*. Several PCR assays have been developed for the detection or serotyping of *A. pleuropneumoniae,* which have seen shown to be more sensitive and specific than serological methods.
The use of PCR allows the amplification of specific DNA sequences and eliminates cross-reactions observed when serological assays are used for typing.

Various PCR assays have been developed for the detection of *A. pleuropneumoniae* in swine. Arbitrarily primed PCR or AP-PCR has been used for serotype identification of *A. pleuropneumoniae*. The advantage of this type of PCR assay is that no prior knowledge of the DNA sequence is required. The use of arbitrary oligonucleotide primers in the amplification of *A. pleuropneumoniae* genomic DNA produces highly specific genomic fingerprints for each serotype. These genomic profiles can be evaluated for genetic relatedness between different species and they can be used to differentiate recognized serotypes of *A. pleuropneumoniae*. This can be useful for serologically cross-reactive strains (Hennessy et al., 1993). Different primers were used for PCR amplification of various regions of the *omlA* gene, which codes for an outer membrane protein that has been cloned and sequenced in all 12 serotypes (Osaki et al., 1997). Analysis of the *omlA* sequences from all 12 serotypes revealed conserved termini and variable middle regions; these could be used to divide the serotypes into distinct groups. The use of primers to the conserved termini and variable middle regions divided the *A. pleuropneumoniae* serotypes into four groups, thereby developing a detection and limited typing system (Townsend et

al., 1998). A comparison of the *omlA* sequence from serotype 8, obtained by Chevallier and Kobisch, and the *omlA* sequence obtained by Gram et al. (2000) revealed differences, indicating the presence of two *A. pleuropneumoniae* serotype 8 strains in circulation. Other PCR assays have utilized the amplification of genes such as the *aroA* gene, (Moral et al., 1999) a dsb-E like element, which produces a thiol:disulfide interchange protein (Chiers et al., 2001) and the *apxIV* gene to develop species-specific assays (Schaller et al., 2001). However sensitive and specific these assays may be, the multiplex PCR assay described by Lo et al. (1998) is both species-specific and serotype-specific. The use of primers to the conserved export and serotype-specific capsular polysaccharide regions enabled the simultaneous detection of *A. pleuropneumoniae* and the identification of serotype 5.

Polymerase Chain Reaction

History

The amazing coincidence regarding Mullis's revelation to develop the idea of PCR was that the science and all the components needed for PCR to work were already in existence and had been for many years. Before the polymerase chain reaction was developed radioactive labeled oligonucleotide probes were commonly used for the detection of specific regions of DNA. This method can and has been used for the diagnosis of hydrops fetalis (Saki et al., 1985). Oligonucleotide probes are small sequences of DNA, that when manufactured will bind to a specific complementary sequence of DNA. It is difficult to study small specific regions of DNA because they are

so large and complex. The use of labeled oligonucleotide probes aids in identifying and isolating smaller DNA fragments that contain the region or gene of interest.

Oligonucleotide probes with the addition of DNA polymerase and dNTP's can produce a complete complementary strand of a DNA fragment. Sanger used this principle to develop dideoxy sequencing. This method can be used to determine the nucleotide sequence of the DNA fragment. Dideoxy sequencing was used as a template for the development of the polymerase chain reaction (Innis et al., 1995).

Kary Mullis is considered the Father of PCR. He developed the idea of PCR in 1983 while driving in his car one evening from San Francisco to Mendocino, California. While an employee at the Cetus Corporation, Mullis conceived a way of controlling a polymerase's action at designated points on a single strand of DNA, thereby expanding on the dideoxy sequencing method. He realized that by harnessing this technology, DNA could be exponentially amplified (www.sunsite.berkeley.edu/biotech/pcr/whatisPCR.html). The idea of amplified synthesis of DNA, which is essentially PCR, was first described in 1977 based on the work of Dr. Har Gobind Khoranas. A postdoctoral fellow, Dr. Kjell Kleppe, in Dr. Khorana's lab performed the first PCR type experiment. Only three or four rounds of amplification were possible however, due to the amount of enzyme needed and the long incubations required in the early days of Molecular Biology. Kary Mullis was unaware of this previous research when he developed the idea of PCR (Templeton, 1992).

Principles of PCR

The polymerase chain reaction is an *in vitro* enzymatic reaction that involves the amplification of specific DNA sequences (Templeton, 1992). The basis of PCR is the use of DNA polymerase. Polymerase is an enzyme that catalyzes the formation and repairs of DNA and can make a copy of the entire DNA in each chromosome. Separation of double-stranded DNA strands allows the DNA polymerase to make a copy by using each strand as a template. DNA polymerase was first discovered by Arthur Kornberg in 1955 and later purified in 1958 (Templeton, 1992). The original DNA polymerase used for PCR was extracted from *E.coli*. Due to the thermolability of the DNA polymerase and the heating required to separate the DNA strands, fresh enzyme has to be added before each cycle. Years later however, a new DNA polymerase was discovered that was able to withstand the high temperatures needed to separate double-stranded DNA. This polymerase is produced by the bacterium *Thermus aquaticus*, which lives in a hot spring in Yellowstone National Park. The DNA polymerase from *T. aquaticus* was purified and cloned and used in the polymerase chain reaction. Due to its ability to survive extended periods of incubation at 95^0 C, fresh enzyme need not be added to each cycle. Currently there are at least nine other commercially available thermostable DNA polymerases. (www.accessexcelence.org/SB/IE/PCR_Xeroging_DNA.html; Templeton, 1992). This modification in performing PCR allowed potential automation of the procedure and it increased the specificity and sensitivity of the reaction.

The components needed to copy DNA are a piece of DNA, large quantities of the four nucleotides A, T, G, and C, primer sequences, and DNA polymerase. The

Polymerase Chain Reaction is carried out in a vial with all the components listed above, and more, at varying temperatures. The starting material or double stranded segment of DNA is denatured, by heating at 95^0 C for one minute. Short sequences of nucleotides called primers, which are complementary to specific sequences on the target sequence bind to the separated DNA strands and are used to prime the copying process. The temperature is lowered to $37\text{-}57^0$ C depending on the G+C content of the primers, allowing them to anneal to their target sequence. The final step in the PCR reaction is to make a complete copy of the template. The temperature is raised to 72^0 C, which is optimal for the DNA polymerase. The polymerase extends the primers in the 3' direction by addition of the nucleotide bases that have been supplied and the new DNA strand is synthesized. One cycle of denaturation, annealing and extension takes 5-10 minutes to complete and a typical PCR will use 30-35 cycles. At the end of one cycle, each strand of DNA has been duplicated. Each piece of new DNA can be used as template in the next cycle and by increasing the number of cycles by 30 or more, the target DNA fragment can be exponentially amplified, approximately 2^n where n is the number of cycles. Eventually the efficiency of the PCR reaction decreases due to accumulation of more primer-template substrate than enzyme available to extend the primer, the stability of the enzyme, competition for substrate by non-specific sequences, and incomplete denaturation of DNA strands due to the increased concentration of product or the limited amount of substrate remaining. At this point the amount of PCR product accumulates in a linear fashion, rather than exponentially (Saki et al., 1988).

Oligonucleotide primers are designed to flank the target DNA of interest. The primers are complementary to specific sequences on the target DNA. Each of the primers binds to the opposite DNA strand. The forward primer binds to the sense strand and the reverse primer binds to the antisense strand. Both primers are extended in the 5'-3' direction and the extension continues until the entire target DNA has been synthesized (Templeton, 1992).

In addition to the oligonucleotide primers, DNA template and Taq polymerase, PCR buffer and deoxynucleotide triphosphates are used in the PCR reaction. The PCR buffer contains KCL, Tris-HCL, and $MgCl_2$. The $MgCl_2$ concentration plays an important role in the specificity and sensitivity of the PCR reaction. If too little is used, no PCR product will be amplified. If too much is used non-specific DNA products will be produced (Templeton, 1992). The specific concentration of $MgCl_2$ should be determined based on each individual reaction. Another parameter that must be determined for each individual PCR reaction is the annealing temperature. The annealing temperature used for PCR is based on the annealing temperature of the primers, which is determined by the G+C content of the primers. The specificity of the PCR reaction is greatly affected by the annealing temperature used. If the annealing temperature is too low, non-specific binding of the primers can occur and non-specific products may be amplified (Innes et al., 1990; Innes et al., 1995).

Due to the increased popularity of PCR it has become automated, which provides convenience and increased reliability when doing a PCR reaction. The time and temperature of the cycling conditions used for PCR are software controlled and can be

programmed into the thermocycler. Commercial kits are also available to aid in the optimization of PCR reactions or to aid in the removal any components, which may inhibit the PCR reaction. However, regardless of the many products available to aid in producing the perfect PCR, care must still be taken to prevent contamination of the PCR reaction. A specified pre-PCR and post-PCR work area should be designated to reduce the risk of contamination. Designated micropipettes should also be used in each of the PCR areas to prevent cross-contamination. Negative and positive controls should always be used when doing a PCR reaction to verify that the concentration of reagents and conditions used are correct and that contamination has not occurred (Innes et al., 1990).

Applications of PCR

PCR has been used for many different applications due to the sensitivity and specificity of the assay as well as the fact that millions of copies of a specific DNA sequence can be amplified in a matter of hours. PCR has become a very useful tool in performing molecular techniques. Some of the more common variations of PCR include the use of nested primers, degenerate primers, inverse PCR, anchored PCR, multiplex PCR, long PCR, arbitrarily primed PCR, and real time PCR. PCR has been used in many fields for diagnostic use, sequencing, forensics, and genetic engineering (Innis et al., 1990; Templeton, 1992; Innis et al., 1995).

The use of PCR for cloning has become prevalent due to the ease in generating micrograms of DNA. This has simplified the process of cloning a single copy gene fragment from most specimens without purifying the DNA. The amount of product

produced from PCR allows direct cloning into the desired vector. Commercial kits are now available for the direct cloning of PCR products into specified vectors, for sequencing, or for gene expression. DNA site-directed mutagenesis and addition of restriction sites is also possible by PCR. Alteration of the complementary nucleotide sequence of one or both of the oligonucleotide primers when designing them, will incorporate the desired mutation or restriction site into the amplified product (Innes et al., 1990; Innes et al., 1995).

DNA sequencing and analysis is another area in which the use of PCR has had great impact. The ability to analyze DNA sequences has provided useful information regarding evolution and the relationship between different species. It has often been difficult to produce enough DNA for analysis, but the use of PCR has overcome this obstacle by its ability to make millions of copies of DNA from a single sequence. Ancient DNA has been amplified by the use of PCR. Previous methods used to analyze ancient DNA involved cloning, and this approach was difficult due to the age and possible modification of the DNA. PCR enabled the isolation of DNA sequences from a few copies of DNA present in extracts. The isolated DNA could be directly sequenced and the sequence analyzed (Innes et al., 1990; Innes et al., 1995).

The detection of genetic sequences is an area in which the use of PCR has become indispensable. The amplified regions of DNA can be sequenced and analyzed for mutations and chromosomal rearrangements. Some of the genetic diseases that have been characterized by PCR include sickle cell anemia, beta-thalassemia, phenylketonuria, and Hemophilia. PCR also has the advantage of being able to identify a specific

individual using DNA from a single hair, semen, blood, saliva, or buccal epithelial cells. Amplification of DNA from these sources has been used for analysis in paternity testing and in forensic science (Innis et al., 1990; Templeton, 1992; Innis et al., 1995).

Viruses are a very important class of pathogens that have been difficult to identify and characterize. The use of PCR has circumvented this problem. Degenerate oligonucleotide primers can be designed to conserved regions of DNA and the viral sequences flanked by these primers are exponentially amplified in PCR. PCR has been used to detect such viruses as Hepatitis B, HIV, HTLV-1, genital human papillomavirus, and the Epstein-Barr virus (Innis et al., 1990; Innis et al., 1995).

PCR and Bacteria

PCR has been used to detect, identify and differentiate many different bacterial species and serotypes. PCR has some advantages over traditional diagnostic assays. Greater specificity was obtained by the use of species-specific and sometimes serotype-specific primers. Cross-reactivity between species or serotypes is reduced compared to serological detection methods. Live or dead organisms can be used as template in a PCR assay, which can be useful for specimens that have been frozen or stored for an extended period of time.

In many cases, PCR can provide results much quicker than other identification methods and culture. This is especially true for the genus *Mycoplasma*, which requires long incubation periods for growth or for Chlymadia, which requires cell culture. The PCR assay is also very sensitive (Caron et al., 2000).

There are several different targets that can be used in a PCR reaction for diagnostic purposes. If no prior knowledge of the sequences to be amplified is available, AP-PCR can be used. The use of arbitrary primers produces a DNA profile of the bacterial genome. This profile can be compared to other species or other serotypes within a species for identification. Pure cultures must be obtained before amplification with PCR. DNA of different concentration or purity or presence of plasmids can dramatically change the profile thereby having negative results with this assay (Hennessey et al., 1993; Innis et al., 1995).

There has been great interest in recent years to use the 16s rRNA gene to detect bacterial pathogens. Some regions of the 16s rRNA have conserved nucleotide sequences while other regions are hypervariable (Tran and Rudney, 1999). Most assays are used to detect a single species, but multiple sets of 16s rRNA primers can be used to detect more than one species in a sample. When RNA is to be used as template, then the first strand of cDNA must be produced with reverse transcriptase and the single stranded cDNA can be directly amplified by PCR (Saki et al., 1985).

Specific DNA sequences, whether they be species or serotype-specific can also be used as a primer target for bacterial identification. The genetic loci encoding the biosynthesis of the O-antigen was used to detect strains O14, O157, and O113 of the Shiga toxigenic-producing *E. coli*. (Paton and Paton, 1999). A species-specific PCR assay was developed for the detection of immunodominant proteins in *Mycoplasma hyopneumoniae*, the primary agent of enzootic pig pneumonia. This assay eliminated cross-reactions of *M. hyopneumonia* with *M. flocculare* and *M. hyorhinis*, previously observed with serological detection methods (Caron et al., 2000).

A serotype-specific PCR assay was also developed to differentiate hemorrhagic-septicemia causing type B strains of *Pasteurella multocida* from other *P. multocida* serotypes (Townsend et al., 1998). Multiplex PCR, which requires multiple primer sets, has also been used for diagnostic assays. Tran and Rudney (1999) reported the use of a multiplex PCR that could simultaneously detect three periodontal pathogens, *Actinobacillus actinomycetemcomitans*, *Bacteroides forsythis*, and *Porphyromonas gingivalis* (Tran and Rudney, 1999). Suzuki also reported the use of multiplex PCR for the identification of *A. actinomycetemcomitans* serotypes a through e (Suzuki, et al., 2001).

Summary

The use of PCR has become overwhelmingly popular for its use in microbial diagnostics as well as in many other areas of science such as, cloning, forensics, detection of genetic disorders etc. The sensitivity and specificity of PCR and its amplification power has resulted in many applications of PCR and many variations on the technique. PCR is becoming more routinely used due to its simplicity and due to the rapidness in performing a PCR reaction. The components needed to perform a PCR reaction are: template, primers, dNTP's, buffer, and Taq polymerase. The potential for contamination when performing a PCR assay must be addressed and specific precautions such as designating a PCR clean room with PCR hood, separate pipetors, filter tips etc., to set up PCR reactions, should be taken to avoid contamination. The use of PCR for bacterial identification has many advantages over other methods. Live or dead organisms can be used as template for PCR, the use of specific primers avoids cross-reactions which

commonly occur with serological detection methods, and due to the amplification power of PCR it is more sensitive than other types of identification.

Chapter 2

Introduction

Actinobacillus pleuropnemoniae is the etiologic agent of swine pleuropneumonia, which is responsible for extensive economic losses each year to the swine industry. The disease is highly contagious and can spread quickly throughout a herd by aerosol transmission. Pigs may develop peracute, acute, or chronic infections following exposure to *A. pleuropneumoniae* (Savoye et al., 2000). In the chronic form, pigs may appear healthy and become carriers that harbor *A. pleuropneumoniae* in the upper respiratory tract. These carriers are the primary source of infection to *A. pleuropneumoniae*-free herds. The capsular polysaccharide protects the pathogen from phagocytosis and complement-mediated killing and determines the serotype specificity of *A. pleuropneumoniae* thereby making the capsule an important antigen for diagnostic purposes (Donachie et al., 1995). *A. pleuropneumoniae* appears to produce a type III capsular polysaccharide consisting of 3 regions. Genes representing regions 1 and 3 have a high degree of homology within and between some species, and encode proteins involved in export of the capsular polysaccharide (*cpx*). In contrast region 2 is serotype-specific and encodes for proteins involved in capsular polysaccharide biosynthesis (*cps*) (Lo et al., 2001). Southern blotting used with a probe to the conserved *cpxD* gene (Ward and Inzana, 1997; Lo et al., 1998) can be used to identify upstream region 2 DNA of any *A. pleuropneumoniae* serotype. There are 12 recognized serotypes of NAD-dependent *A. pleuropneumoniae,* each of which synthesizes a unique capsular polysaccharide (Nicolet, 1988; Inzana and Mathison, 1997; Nielsen et al., 1997). Therefore early detection and

identification of the infectious serotype is important in order to begin proper treatment and to control spread of disease in the herd (Rosendal et al., 1981). Serotypes vary in their degree of virulence and by geographic location. Serotypes 1, 5, and 7 are most prevalent in North America, serotypes 1, 7, and 12 are dominant in Australia (Zang et al., 2000), serotype 3 is common in Quebec and Ireland (Dubreuil et al., 2000), and serotypes 2, 8, and 9 are more common in Europe (Mittal et al., 1992; Belanger et al., 1995; Gram and Ahrens, 1998).

Standard identification of *A. pleuropneumoniae* requires culture and serotyping. Serotyping methods such as latex agglutination, tube agglutination, ring precipitation, and agar gel immunodiffusion have been used to detect *A. pleuropneumoniae*. Some typing methods may be problematic due to antigenic cross-reactivity between heterologous serotypes and identifying occasional untypeable isolates. Cross-reactions between serotypes 1, 9 and 11, 4 and 7, and 3, 6 and 8 have been reported and are most likely due to common epitopes on the O-side chain of the lipopolysaccharide (LPS) and membrane proteins (Paradis et al., 1994).

DNA amplification by PCR is an alternative method currently being used to identify *A. pleuropneumoniae* and may be more sensitive than culture because viable organisms are not required. Various PCR assays have been developed for the identification of *A.pleuropneumoniae* (Hennessey et al., 1993; Moral et al., 1999; Chiers et al., 2001). However, most of these assays do not simultaneously detect *A. pleuropneumoniae* and identify the serotype. Jessing et al. (2002) has reported the use of a multiplex PCR assay for the identification of serotypes 2, 5, and 6. Lo et al. (1998) has

previously reported development of a multiplex PCR assay to identify A. *pleuropneumoniae* serotype 5 based on the simultaneous amplification of conserved genes required for capsular polysaccharide export and serotype-specific genes required for serotype 5 capsular polysaccharide biosynthesis. We now describe an expansion of the previous multiplex PCR assay to include the identification of serotypes 1, 2, and 8, and the partial characterization of the biosynthesis genes in serotype 8.

Materials and Methods

Bacterial strains, plasmids, and growth conditions.

The bacterial strains and plasmids used in this study are shown in Table 1. All strains were grown at 37^0 C. *A. pleuropneumoniae* strains were grown on Brain Heart Infusion (BHI) agar (Difco Laboratories, Detroit, MI) supplemented with β-NAD (5μg/ml; Sigma Chemical Co., St. Louis, MO*).* *Mannheimia haemolytica* A1 was grown on BHI. *E.coli* transformants were grown in Luria-Bertani (LB) broth containing 10 g/L NaCl for both routine cultivation and extraction of plasmids, and supplemented with ampicillin (100 μg/ml). For blue-white screening of pBluescript ligations, *E.coli* DH5α cells were spread on LB plates with 40 μl of 20 mg/ml X-gal and 4 μl of 200 mg/ml IPTG (Sambrook and Russel, 2001)

Table 1. Bacterial strain or plasmid and their sources

Species	Serotype	Strain	Source
A. pleuropneuomoniae	1	4074	ATCC
A. pleuropneuomoniae	1	4045	ATCC
A. pleuropneuomoniae	2	27089	ATCC
A. pleuropneuomoniae	3	27090	ATCC
A. pleuropneuomoniae	4	33378	ATCC
A. pleuropneuomoniae	5	J45	B. Fenwick
A. pleuropneuomoniae	6	33590	ATCC
A. pleuropneuomoniae	7	WF83	ATCC
A. pleuropneuomoniae	8	405	K Mittal
A. pleuropneuomoniae	9	13261	Nicolet
A. pleuropneuomoniae	10	D13039	K. Mittal
A. pleuropneuomoniae	11	56153	M. Ryder
A. pleuropneuomoniae	12	8329	K. Mittal
A. pleuropneumoniae		Field Isolates	Rollins Diagnostic Lab, NC
M. haemolytica		A1	N. Sriranganathan
H. influenzae type b		Eagen	Porter Anderson
H. parainfluenzae		Field Isolate	Rollins Diagnostic Lab, NC
H. ovis		Field Isolate	Rollins Diagnostic Lab, NC
E. coli Chemically Competent DH5α			Life Technologies Inc., Rockville, MD
Plasmids			
pBluescript II SK(+/-) phagemid - Cloning vector 2.9kb Amp^r			Promega
pJSAp81 - 3.6kb ClaI fragment of Ap serotype 8 cloned into pBSK II (+/-) Amp^r			This work
pJSAp82 - 1.8kb EcoRV fragment of Ap serotype 8 cloned into pBSK II (+/-) Amp^r			This work
pJSAp83 - 2.0kb EcoRV fragment of Ap serotype 8 cloned into pBSK II (+/-) Amp^r			This work

Lung specimens.

Lung tissue samples were taken from pigs that had been intratracheally challenged with 1.7 x10^7 colony forming units (CFU's) of *A. pleuropneumoniae* serotype 1. Lung tissue samples were taken at necropsy and stored at -20^0 C.

Recombinant DNA methods.

Bacterial genomic DNA was isolated by use of a QIAamp DNA mini kit, following the manufacturer's recommendations (Qiagen, Valencia, CA). Large-scale preparations of plasmid DNA were isolated by alkaline lysis, and purified with Qiagen-tip 100 columns. Small-scale preparations of plasmid DNA were isolated using a Qiaprep spin Miniprep kit (Qiagen). Restriction fragments selected for cloning were eluted from 0.7% agarose gels using the Qiagen Gel Extraction Kit. Following heat shock, recombinant plasmids were transformed into *E.coli* and colonies screened by colony hybridization using the *cpxD*, *cps8A*, and *cps8CD* DNA probes. Positive clones were purified and sequenced.

DNA Hybridization.

Molecular Biology techniques were performed as described in Molecular Cloning (Sambrook and Russel, 2001). DNA fragments to be used as probes were amplified by PCR and labeled by the random primer method using a Genius system nonradioactive labeling and detection kit (Boehringer Mannheim Corp. Indianapolis, IN). DNA hybridizations were performed at 60^0 C with the *cpxD* probe, (Ward and Inzana, 1997) at 59^0 C with the *cps8A* probe, and at 49^0C with the *cps*8*CD* probe, respectively, in

solutions containing 5X SSC. These temperatures were determined optimal for use with the respective probes. The membranes were washed and developed as described by the manufacturer.

Multiplex PCR Sample Preparation.

DNA from whole bacterial cells was extracted by suspending the cells in 100 µl of sterile water followed by boiling at 100^0 C for 10 minutes. The cell debris was sedimented by centrifugation at 8000 x g for 10 minutes and 5 µl of the supernatant containing the DNA was used as template in the PCR. Bacterial DNA was extracted from lung tissue samples by slicing the tissue into 2-mm long sections, mashing, and vortexing the sections in 1 ml of sterile water. The samples were then boiled at 100^0 C for 10 minutes. The tissue samples were sedimented by centrifugation at 10,000 x g for 10 minutes and 5µl of the supernatant was used as DNA template for PCR.

Latex Agglutination Test.

The latex agglutination test was used to identify serotypes 1, 5, and 7 from *A. pleuropneumoniae* field isolates as previously described by Inzana (1995).

DNA Primers.

Oligonucleotide primers were selected by using DNA Star Primer Select software (Madison WI). Primers *cpxAF*, *cpxAR*, $cpxU_1$, $cpxL_1$, *Ap5C*, and *Ap5D* were designed from the conserved capsular polysaccharide export region of *A. pleuropneumoniae*

serotype 5; Forward and reverse primers, *Ap1U*$_1$, *Ap1L*$_1$ and *Ap1L*$_2$, *Ap2U*$_1$ and *Ap2L*$_1$, *Ap5A*, and *Ap5B*, *Ap8U*$_1$ and *Ap8L*$_1$ were designed from the serotype–specific capsular polysaccharide biosynthesis regions of *A. pleuropneumoniae* serotypes 1, 2, 5, and 8, respectively. Primers *MHU* and *MHL* were designed from the *wbrA* and *wbrB* genes, respectively from region 3 of *M. haemolytica* A1 (Table 2). The primers were selected based on the following parameters: primer length, product length, product location, minimal hairpin formations, dimer formations, and annealing temperature.

Table 2. Primer sequences used for multiplex PCR

	Name	Sequence	Primer size(bp)	Product Size(bp)
Forward Primer Export Region	cpxAF	5'- TAGAACCTTGTA AGCCTCGTCCATA-3'	24	489
Reverse Primer Export Region	cpxAR	5'-CGTTTGTTAAGT GGTGTTGAGC-3'	22	
Forward Primer Export Region	$cpxU_1$	5'-GGAATCGCTACA GTTACCCAAAAT-3'	24	881
Reverse Primer Export Region	$cpxL_1$	5'-ACACCGGAAGC GATTCAGTCTCA-3'	23	
Forward Primer Export Region	Ap5 C	5'-TGGCGATACCG GAAACAGAGTC-3'	22	715
Reverse Primer Export Region	Ap5 D	5'-GCGAAAGGCTATG GTATGGGTATGG-3'	24	
Forward Primer Biosynthesis Region	$Ap1U_1$	5'-AGTGGCTGGAT GAGACGAGAC-3'	21	1603
Reverse Primer Biosynthesis Region	Ap1 L_1	5'- AGGCTTGCCC ACCATTTTC-3'	19	1000
Reverse Primer Biosynthesis Region	$Ap1L_2$	5'-TAGTTTGTTATG GTATTTCTGTA -3'	23	
Forward Primer Biosynthesis Region	$Ap2U_1$	5'-CGCAGCCGGACAA AAACAAATACACG-3'	26	1725
Reverse Primer Biosynthesis Region	$Ap2L_1$	5'-CACCCCATGAATC GACTGATTGCCAT-3'	26	
Forward Primer Biosynthesis Region	Ap5A	5'-TTTATCACTATCA CCGTCCACACCT-3'	25	1100
Reverse Primer Biosynthesis Region	Ap5B	5'-CATTCGGGTCTT GTGGCTACTAA-3'	23	

Forward Primer Biosynthesis Region	Ap8 U_1	5’-AACGGCTTTTGAAC AACTTTATTTATTT-3’	28	977
Reverse Primer Biosynthesis Region	Ap8 L_1	5’-TTCATTCCTAAACT CCGTATTGTCA-3’	25	
Forward Primer Export Region	MHU	5’-AATTTAGTTGCACCG CTTTCTTTAGTAGTC’-3’	30	970
Reverse Primer Export Region	MHL	5’-GACCTCTTTTTGCT CACTTTCTAACCA–3’	27	

PCR.

All PCR reactions were performed in a total volume of 50 µl and master mix concentrations and cycling conditions were optimized based on the serotype. One to two micrograms of DNA template was used for each reaction. The final volume of each master mix contained 1X PCR buffer (Fisher Scientific, Pittsburg, PA), 200mM dNTP's, and 2U Taq polymerase. For identification of *A. pleuropneumoniae* serotype 1 (Ap 1), the PCR assay contained a final concentration of 3 mM $MgCl_2$, 20 pmol each of serotype-specific primers, and 10 pmol each of conserved export primers. For identification of *A. pleuropneumoniae* serotype 2 (Ap 2), the PCR assay mix contained a final concentration of 2 mM $MgCl_2$ and 10 pmol each of conserved export and serotype-specific primers. The *A. pleuropneumoniae* serotype 5 (Ap 5) PCR assay mix contained a final concentration of 2 mM $MgCl_2$ and 10 pmol each of conserved export and serotype-specific primers. For identification of *A. pleuropneumoniae* serotype 8 (Ap 8) the assay mix contained a final concentration of 3 mM $MgCl_2$ and 10 pmol each of Ap8 specific primers and 20 pmol each of conserved export primers. All multiplex PCR reactions were performed in an Omnigene Thermal Cycler (Hybaid Unlimited, England). Cycling conditions for each of the different PCR reactions are shown in Table 3. All PCR reactions were replicated a minimum of 4 times to determine the optimal concentrations and conditions. Positive and negative controls were used when optimizing PCR reactions. PCR products were separated by electrophoresis and visualized under UV light. The identity of the PCR product was confirmed by sequencing. For detection of region 3 DNA in *A. pleuropneumoniae* serotype 2 by PCR, 1X PCR buffer, 200 mM dNTP's,

10 pmoles each of *MHU* and *MHL* primers, and 2U of Taq polymerase were used and carried out as described above. DNA amplification and analysis was carried out as described above.

TABLE 3. Cycling conditions for PCR reactions

Ap1 specific pimers
1 cycle:
95^0C 3 minutes Denaturation
33 cycles:
95^0C 1 minute Denaturation
56^0C 1 minute Annealing
72^0C 2 minutes Extension
1 cycle:
72^0C 10 minutes Final Extension

Ap2 specific primers
1 cycle:
95^0C 3 minutes Denaturation
35 cycles:
95^0C 1 minute Denaturation
60^0C 1 minute Annealing
72^0C 2 minutes Extension
1 cycle:
72^0C 10 minutes Final Extension

Ap5 specific pirmers
1 cycle:
95^0C 3 minutes Denaturation
31 cycles:
95^0C 1 minute Denaturation
54^0C 1 minute Annealing
72^0C 1 minute Extension
1 cycle:
72^0C 8 minutes Final Extension

Ap8 specific primers
1 cycle:
94^0C 3 minutes Denaturation
29 cycles:
94^0C 30 seconds Denaturation
60^0C 30 seconds Annealing
72^0C 40 seconds Extension
1 cycle:
72^0C 10 minutes Final Extension

DNA sequencing and analysis.

Templates for DNA sequencing were constructed by subcloning fragments generated from specific restriction sites within *A. pleuropneumoniae* serotype 8 into pBluescript II SK+/-. Custom Synthetic Oligonucleotide primers were obtained from Genosys Biotechnology and used for sequencing. Templates were sequenced at the Core Sequencing Laboratory Facility at the Virginia Bioinformatics Institute (Virginia Tech) using standard methods on an ABI 3100 capillary sequencer and using applied Biosystems Big Dye (version 2.0) Terminator chemistry. Sequence analysis was performed using the DNA Star Analysis Computer package of programs (DNA Star, Madison, WI). The identity of the cloned fragments was detemined based on homology to other capsule biosynthesis proteins. Database searches were performed using the BLAST program provided by the National Center for Biotechnology Information (NCBI).

RESULTS

Cloning of the serotype 8 capsule biosynthesis genes

A 480 bp PCR fragment from within a portion of the *cpxD* gene of *A. pleuropneumoniae* serotype 5 was used as a probe with *Cla*I-digested *A. pleuropneumoniae* serotype 8 genomic DNA to identify the homologous *cpx8D* gene and part of the adjacent serotype–specific *cps* DNA. A 3.7 kb fragment of serotype 8

genomic DNA hybridized with the probe at 60^0 C. This fragment was cloned into pBluescript and the clone designated pJSAp81 was sequenced. DNA sequencing revealed that the pJSAp81 insert contained 843 bp of the homologous *cpx8C* gene, the entire *cpx8D* gene, one complete and one incomplete unidentified ORF that were transcribed in the opposite direction. The location and orientation of these unidentified ORF's relative to the *cpxD* gene were consistent with the location of the capsule biosynthesis genes from serotypes 1, 2, 5, and other bacterial species that express type III capsules (Ward and Inzana, 1997; Lo et al., 2001). These two unidentified ORF's were putatively designated *cps8A* and *cps8B*. The entire *cps8A* gene was present in the pJSAp81 insert, while only 233bp of the cps8B gene was present. A digoxigenin-labeled 430 bp PCR fragment amplified from 365 bp of the 3' end of *the cps8A* gene and encompassing the first 5 bp of the *cps8B* gene was used as a probe. The probe hybridized with a 1.6 kb EcoRV digested fragment at 59^0 C. The 1.6 kb fragment was cloned into pBluescript and the clone was sequenced and designated pJSAp82. DNA sequencing revealed that the pJSAp82 insert contained 649 bp of sequence that overlapped with pJSAp81, but contained a new ORF, which was designated *cps8C*. In the pJSAp82 insert, 746bp of the *cps8C* gene was present. A 580 bp fragment was amplified by PCR from the 3' terminal end of the last 181 bp of the *cps8C* gene and the first 394 bp at the 5' end of the *cps8D* gene. The PCR fragment was digoxigenin labeled and used as a probe. A 2.0 kb fragment of *Eco*RV-digested serotype 8 genomic DNA hybridized with this probe at 49^0 C. The 2.0 kb fragment was cloned into pBluescript, sequenced and designated pJSAp83. The pJSAp83 insert contained the remaining 405 bp of the *cps8C* gene, the entire 998 bp of the *cps8D*

gene and 591bp of sequence, which may be a new open reading frame. (Figure 1) An additional fragment downstream of the *cps8D* gene has been identified by PCR and may contain additional *cps8* sequence.

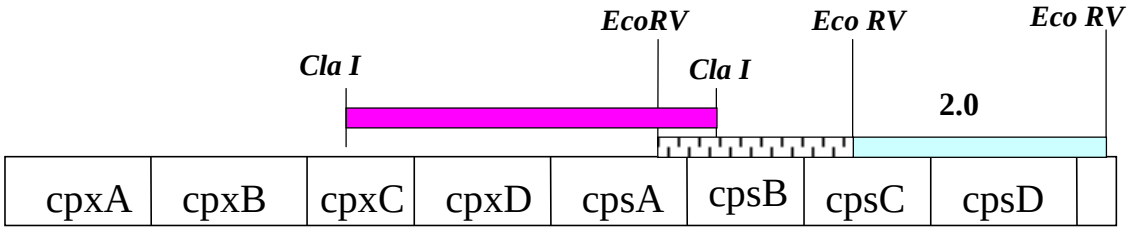

Figure 1. Cloned serotype 8 capsular polysaccharide biosynthesis genes

The majority of the *cps* genes from *A. pleuropneumoniae* serotype 8 did not reveal any substantial homology at the nucleotide level with other sequences in the nucleotide databases at NCBI. The exception was *cps8B*, which had 82% identity with *tagD* from the techoic acid biosynthesis locus in *Bacillus subtilis*. These results are similar to BLAST searches conducted on the *cps* genes of *A. pleuropneumoniae* serotype 2, which also showed similarity to the *tagD* from *B. subtilis* (personal communication). At the amino acid level, BLAST searches revealed 29% identity between Cps8A and a techoic acid biosynthesis protein from *Lactococcus lactis*. Cps8A also had 25% identity to a putative glycosyl transferase from *Streptomyces coelicolor*. Cps8B had 70% and 68% identity with glycerol 3- phosphate cytidyltransferases from *B. subtilis* and *Listeria monocytogenes* respectively. Cps8C had local areas of homology with 24% overall identity with a fukutin protein from *Homo sapiens*, and homology to a hemolysin erythrocyte lysis protein from *Prevotella intermedia*. BLAST searches with Cps8D revealed 38% local identity to a TRP or transient receptor protein from *Clostridium acetobutylicum* but overall had 35% identity to a glycero phosphotransferase from *Streptococcus pneumoniae* and 30% identity to a TagF protein from *Listeria innocua*.

Identification and Characterization of Region 3 in *A. pleuropneumoniae* serotype 2

As previously stated type III capsular polysaccharides consist of 3 regions. Regions 1 and 3 are involved in export of the capsular polysaccharide and are conserved with-in and between species. Region 2 is serotype-specific and involved in capsular

polysaccharide biosynthesis. In *A. pleuropneumoniae* serotype 5, regions 1 and 2 have previously been cloned and sequenced (Ward and Inzana, 1997). Region 1 has also been detected in all 12 *A. pleuropneumoniae* serotypes by PCR (Lo et al., 1998). Until now, region 3 has not been identified in any of the 12 *A. pleuropneumoniae* serotypes.

M. haemolytica A1 is a bovine pathogen and also produces a capsular polysaccharide whose capsule gene cluster can be divided into 3 regions similar to *A. pleuropneumoniae*. Region 3 of *M. haemolytica* has recently been characterized and the two ORF's identified were designated *wbrA* and *wbrB* (Lo et al., 2001). These two ORF's are proposed to be involved in substitution of phospholipids on the capsular polysaccharide. The *wbrA* and *wbrB* capsular polysaccharide cluster is homologous to the *kpsC* and *kpsS, lipA* and *lipB,* and *phyA* and *phyB* capsular polysaccharide clusters in *E.coli* K5, *N. meningitidis,* and *P. multocida* respectively (Lo et al., 2001).

The PCR assay using primers *MHU* and *MHL* amplified a 970 bp fragment in *M. haemolytica* containing a portion of both the *wbrA* and *wbrB* genes. Under very low stringency the PCR assay amplified a faint 970 bp fragment in *A. pleuropneumoniae* serotype 2. Gel plugs were made from this faint 970 bp fragment and re-amplified by PCR using the same primers. The re-amplified fragment from *A. pleuropneumoniae* serotype 2 was purified by gel extraction and sequenced. The sequence obtained from the Ap2 fragment amplified and sequenced with *MHU,* showed 85% identity to the PhyA protein of *P. multocida* and 48% identity to the LipA protein from *Neisseria meningitidis*. Sequence obtained from the Ap2 fragment amplified and sequenced with *MHL*, showed 72% identity to the PhyB protein of *P. multocida*.

Standardization and optimization of the multiplex PCR

The multiplex PCR assay specific for *A. pleuropneumoniae* serotype 5 was expanded to include identification of *A. pleuropneumoniae* serotypes 1, 2, and 8. Two additional sets of primers were designed from the sequenced DNA of the *A. pleuropneumoniae* serotype 5 capsular polysaccharide export region. Primer sets *Ap5C* and *Ap5D* have been described (Lo et al., 1998). Primers *cpxU*$_1$ and *cpxL*$_1$, were designed to amplify a fragment containing portions of both the *cpxC* and *cpxD* genes to increase specificity. Primers *cpxAF* and *cpxAR* were later designed due to non-amplification of the *cpxCD* fragment in serotype 4. These primers would amplify a fragment containing the *cpxA* gene. Figure 2 shows where the primer sets should amplify.

Amplification of J45 genomic DNA with primers *cpxU*$_1$ and *cpxL*$_1$ or *cpxAF* and *cpxAR* resulted in production of a 880 bp, or 489 bp band, respectively. To verify the conserved nature of the region from which these primers were designed, the *cpxAF* and *cpxAR, and cpxU*$_1$ and c*pxL*$_1$ primer sets were used to amplify DNA from whole cells of the reference strains of all twelve *A. pleuropneumoniae* serotypes. Primers *cpxU*$_1$ and *cpxL*$_1$ amplified a single 880 bp fragment, in all serotypes except serotype 4. Primers *cpxAF* and *cpxAR* amplified a 489bp fragment in all twelve serotypes including serotype 4.

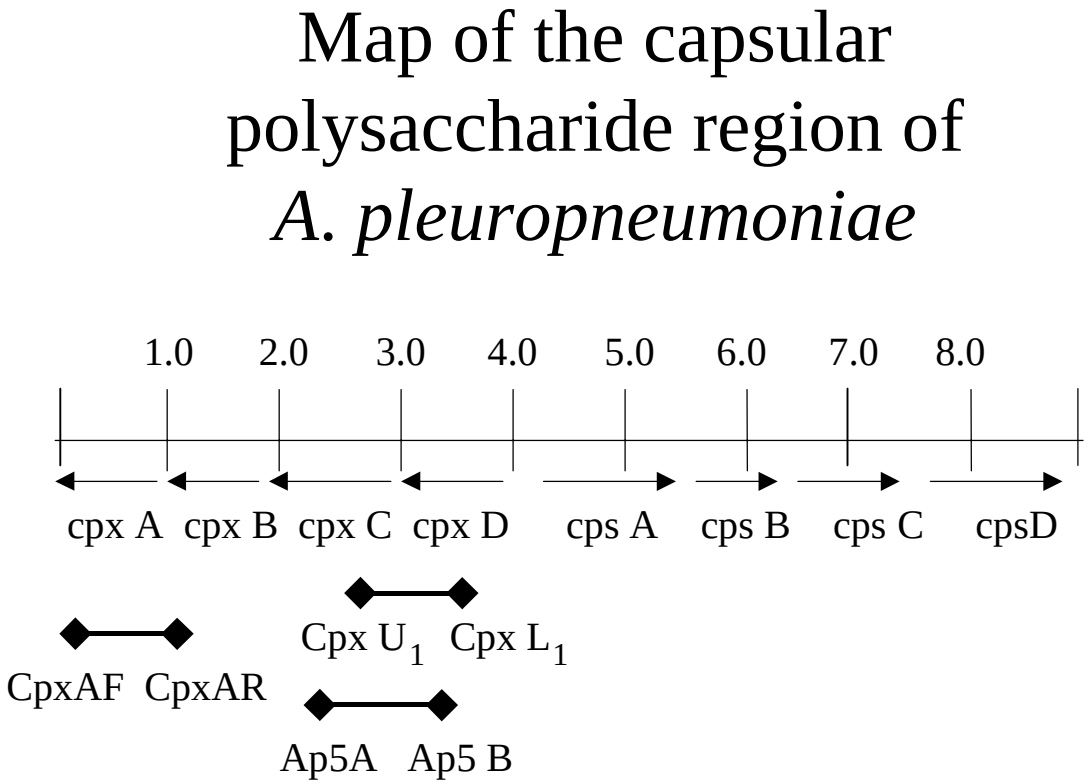

Figure 2. Map of the capsular polysaccharide region of *A. pleuropneumoniae* and location of the conserved *cpx* primers used for PCR

Primers specific to the *cps* regions of *A. pleuropneumoniae* serotypes 1, 2, and 8 were designed. Primers $Ap1U_1$ and $Ap1L_1$, $Ap1U_1$ and $Ap1L_2$ were designed and used to amplify a 1.0 kb or 1.6 kb, fragment in *A. pleuropneumoniae* serotype 1, and $Ap2U_1$ and $Ap2L_1$, and $Ap8\ U_1$ and $Ap8\ L_1$ were designed to amplify 1.7 kb and 970 bp fragments, respectively, in *A. pleuropneumoniae* serotypes 2 and 8. These serotype-specific primers were used in conjunction with the $cpxU_1$ and $cpxL_1$ or *cpxAF* and *cpxAR* primers for the multiplex PCR. Figure 3 shows where the primer sets should amplify.

Map of the capsular polysaccharide region of *A. pleuropneumoniae*

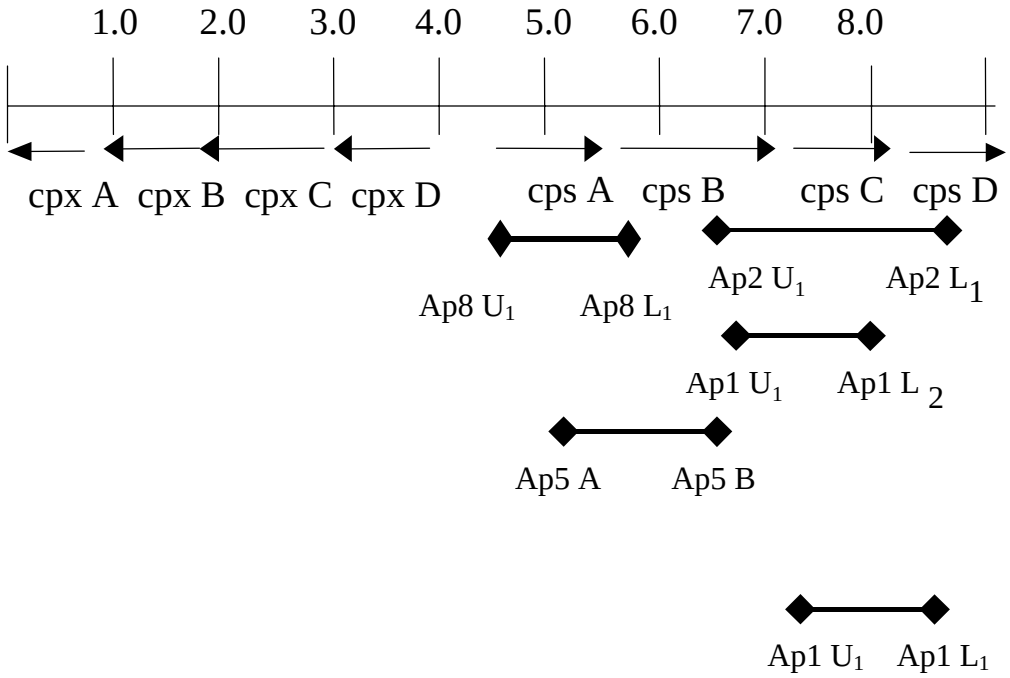

Figure 3. Map of the capsular polysaccharide region of *A. pleuropneumoniae* and location of the serotype-specific *cps* primers used for PCR

Amplification of Ap1 4074 genomic DNA with the *Ap1U*$_1$ and *Ap1L*$_2$ (*cps*) and *cpxU*$_1$ and *cpxL*$_1$ (*cpx*) primers resulted in detection of both the 1.6 kb and 880 bp bands, respectively. Amplification of Ap2 genomic DNA using the *Ap2U*$_1$ and *Ap2L*$_1$ (*cps*) and *cpxU*$_1$ and *cpxL*$_1$ (*cpx*) primers resulted in detection of both the 1.7 kb and 880 bp bands, respectively. Amplification of Ap5 J45 genomic DNA with *cpx* and *cps* primers was previously described (Lo et al., 1998). Figure 4 shows the amplification of DNA from all 12 serotypes of *A. pleuropneumoniae* using *A. pleuropneumoniae* serotypes 1, 2, and 5 specific primers and their corresponding *cpx* primers. Amplification of Ap8 genomic DNA with *Ap8U*$_1$ and *Ap8L*$_1$ (*cps*) and *cpxAF* and *cpxAR* (*cpx*) primers resulted in detection of both the 970 bp and 480 bp bands, respectively. Figure 5 shows the amplification of serotypes 1-12 using *A. pleuropneumoniae* serotype 8 specific primers and the conserved export primers.

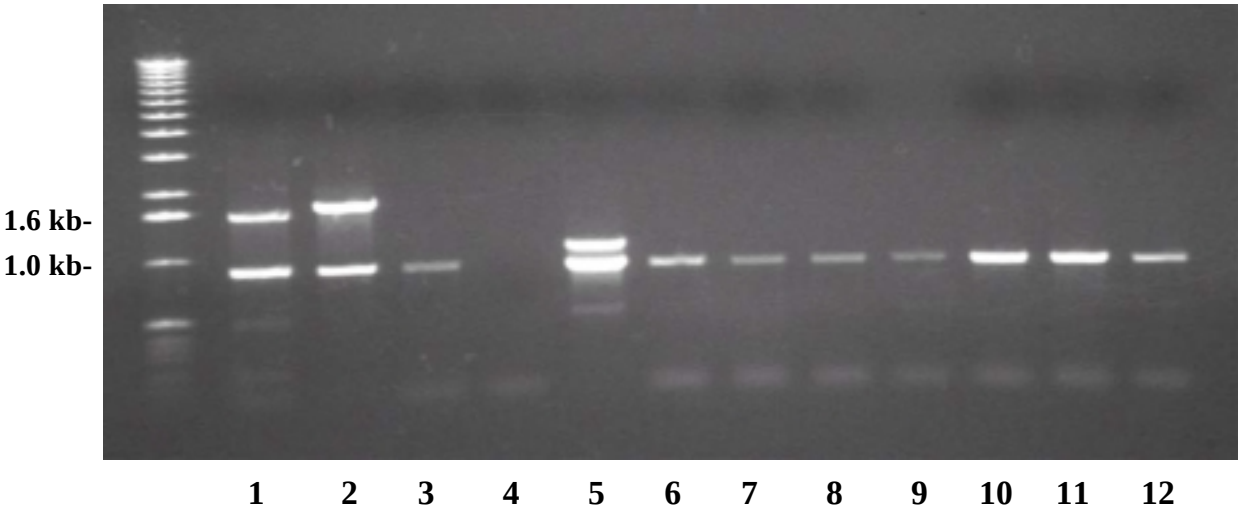

Figure 4. Agarose gel electrophoresis of *A. pleuropneumoniae* serotypes 1- 12. 1- kb ladder, lanes 1, 3, 4 and 6-12, amplification of serotypes 1, 3, 4 and 6-12 with primers $Ap1U_1$ and $Ap1L_2$ and $cpxU_1$ and $cpxL_1$, lane 2, amplification of serotype 2 with $Ap2U_1$ and $Ap2L_1$ and $cpxU_1$ and $cpxL_1$, lane 5, amplification of serotype 5 with Ap5A and Ap5B and Ap5C and Ap5D.

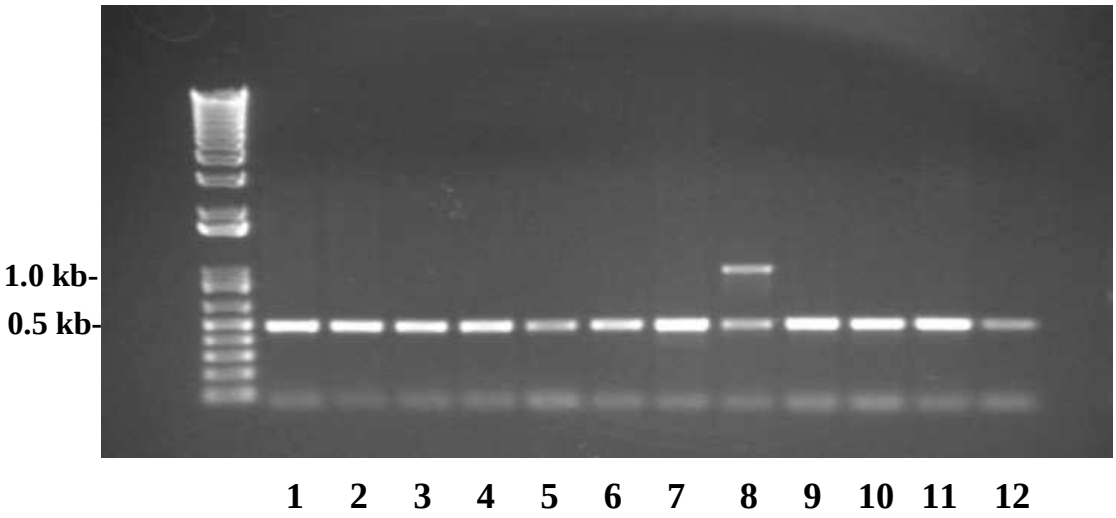

Figure 5. Agarose gel electrophoresis of *A. pleuropneumoniae* serotypes 1-12. 1-kb ladder, lanes 1-12, Amplification of serotypes 1-12 with cpxAF and cpxAR and $Ap8U_1$ and $Ap8L_1$ primers.

The multiplex PCR reactions were optimized by replicating assays and varying the concentrations of magnesium chloride and annealing temperatures. The magnesium chloride concentration was varied between 1 mM and 5 mM. When using Ap1-specific primers, magnesium chloride concentrations lower than 3mM eliminated bands. A 3 mM $MgCl_2$ concentration was determined to be optimum. When using Ap2- specific primers, concentrations of magnesium chloride greater than 3mM produced non-specific bands in all serotypes. A 2 mM $MgCl_2$ concentration was determined to be optimum for the amplification of Ap2 products. For amplification of Ap8 DNA products, a 3 mM $MgCl_2$ concentration was determined to be optimum. When 2 mM $MgCl_2$ was used, the 480 bp *cpx* band was not amplified in Ap8. The annealing temperatures for each primer set were varied 3^0 higher and lower than the average of the two different annealing temperatures for the specific primer set. Overall, decreasing the annealing temperature increased the number of non-specific bands amplified. As the annealing temperature was raised non-specific bands were eliminated, in addition to the *cpx* and *cps* bands. The optimum annealing temperature for Ap1 PCR was 56^0. Temperatures higher than 56^0 eliminated the *cpx* band in Ap1. For the Ap2 and Ap8 PCR, 60^0 was determined to be the optimum annealing temperature and temperatures above this eliminated the *cpx* band from Ap2 and Ap8. The Ap5 PCR was previously optimized (Lo et al., 1998).

Assay of Clinical Samples

Primers specific to the *cpx* region were used to detect *A. pleuropneumoniae* in lung tissue samples challenged with serotype 1. Figure 6 shows amplification of DNA from lung

tissue samples using *cpxU*$_1$ and *cpxL*$_1$ primers. Ap1 specific primers that amplified a 1.0 kb fragment, amplified serotype 1 DNA from lung tissue samples in swine challenged with *A. pleuropneumoniae* serotype 1. Figure 7 shows amplification of serotype 1 DNA from lung tissue samples using *A. pleuropneumoniae cpxU*$_1$ and *cpx L*$_1$ and serotype 1 *Ap1U*$_1$ and *Ap1L*$_1$ primers. Figure 8 shows amplification of serotype 1 DNA from lung tissue samples using *A. pleuropneumoniae* serotype 1 primers *Ap1 U*$_1$ and *Ap1 L*$_2$ that amplified a 1.6 kb fragment.

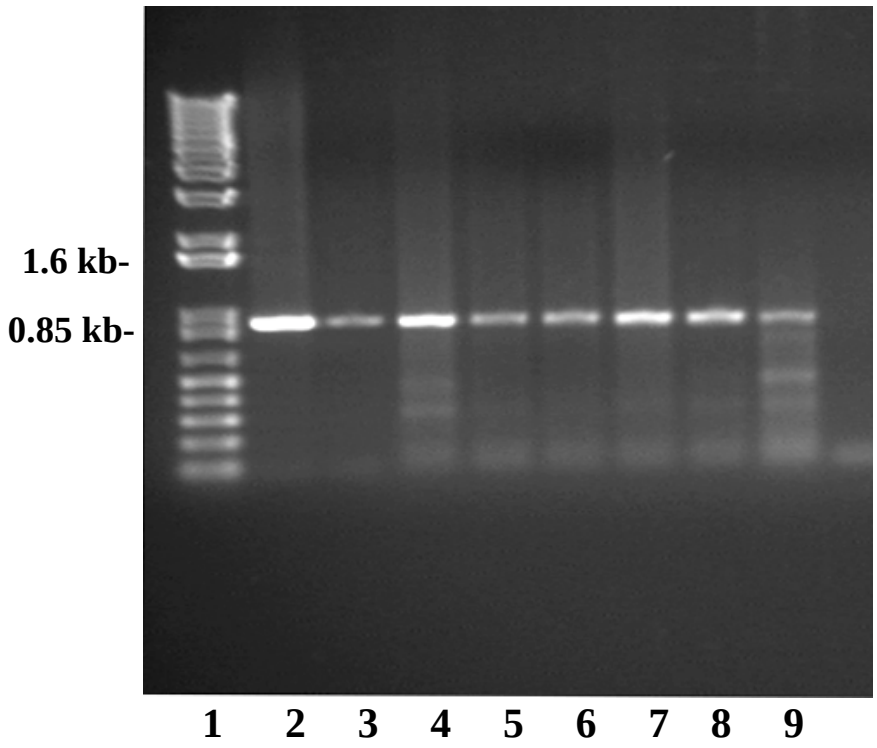

Figure 6. Agarose gel electrophoresis of *A. pleuropneumoniae* amplified from lung tissue samples. Lane 1, 1-kb ladder, lanes 2-9, Ap serotype 1 genomic DNA and samples 537, 96, 552, 564, 532, 332 and 99 amplified with $cpxU_1$ and $cpxL_1$.

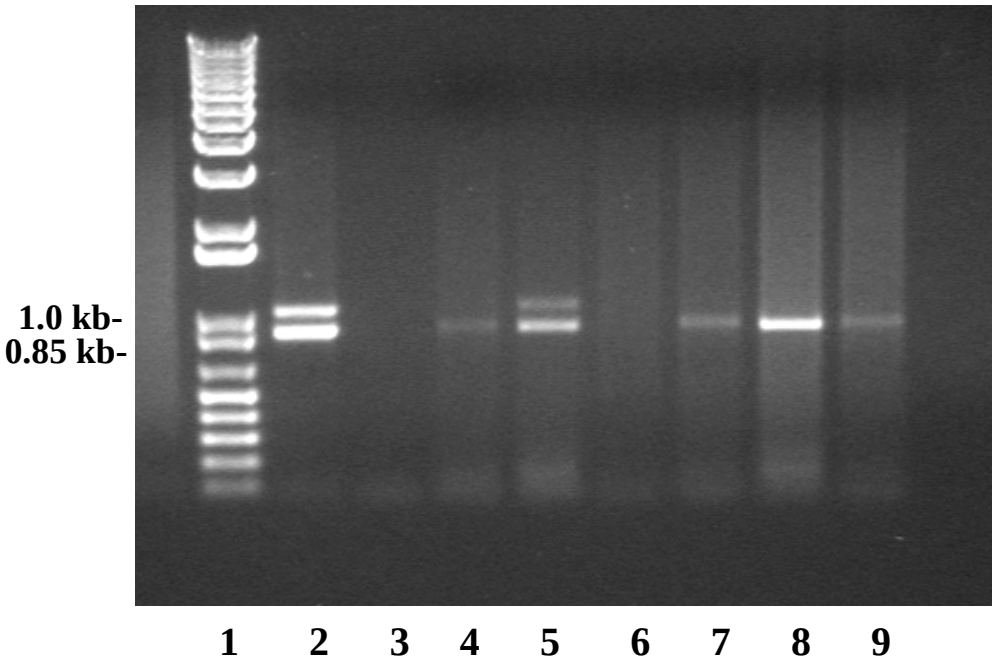

Figure 7. Agarose gel electrophoresis of *A. pleuropneumoniae* lung tissue samples amplified with both $cpxU_1$ and $cpxL_1$ and $Ap1U_1$ and $Ap1L_1$ primers. Lane 1, 1kb ladder, lane 2, positive control, lane 3, negative control, lanes 4-9, samples 99, 232, 564, 252, 552, and 96.

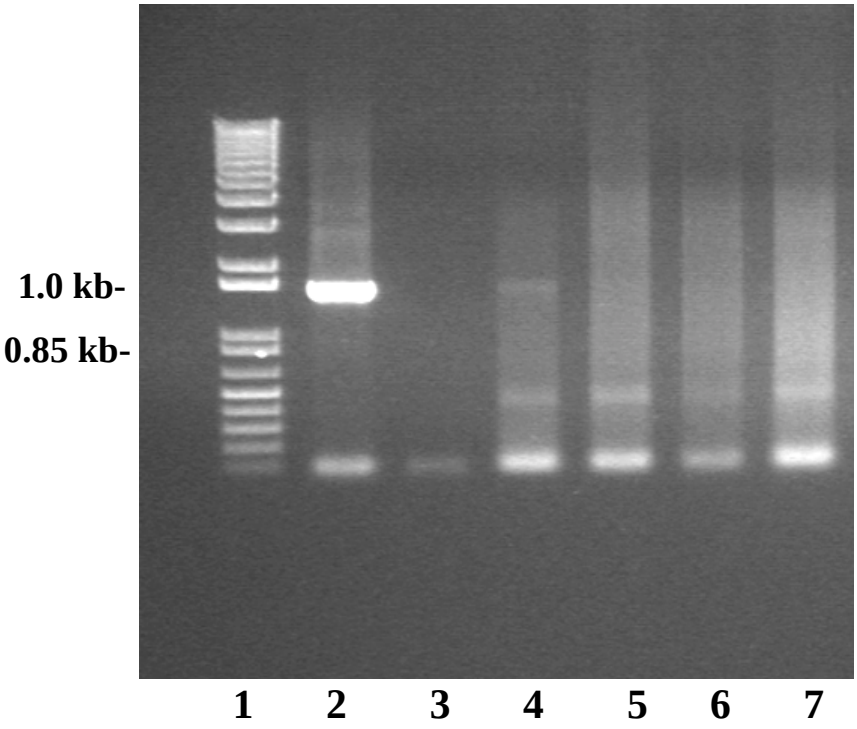

Figure 8. Agarose gel electrophoresis of *A. pleuropneumoniae* amplified from lung tissue samples using $Ap1U_1$ and $Ap1L_2$ cps primers. Lane 1, 1-kb ladder, lane 2, Ap1 positive control, lanes 3-7, samples 99, 564, 532, 332 and 96.

Assay Specificity

The use of *A. pleuropneumoniae* primers *cpxU*$_1$ and *cpxL*$_1$ and serotype- specific primers *Ap1U*$_1$ and *Ap1L*$_2$ amplified a 880 bp and 1.6 kb fragment in *A. pleuropneumoniae* serotype 1 only. Neither the 880 bp nor the 1.6 kb fragment were amplified in the negative control or in any of the non- *A. pleuropneumoniae* isolates. The 880 bp fragment was amplified in K17-C, which is an acapsular mutant of *A. pleuropneumoniae* serotype 5. Figure 9 shows the PCR products amplified from the Ap and non-Ap isolates with the primers *cpx U*$_1$ and *cpxL*$_1$ and *Ap1U*$_1$ and *Ap1 L*$_2$.

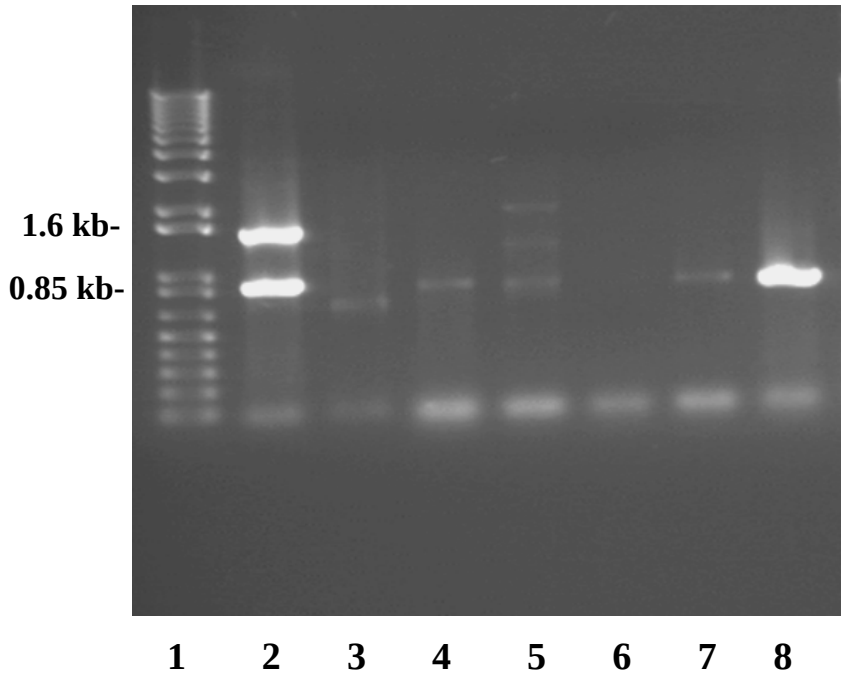

Figure 9. Agarose gel electrophoresis of *A. pleuropneumoniae* serotype 1, Ap and non- Ap isolates amplified with $cpxU_1$ and $cpxL_1$ and $Ap1U_1$ and $Ap1L_2$. Lane 1, 1 kb ladder, lane 2 *A. pleuropneumoniae* serotype 1, lane 3 negative control, lanes 4-8, *H. ovis, H. influenzae, H. parasuis, H. paragallinarium*, and K17-C (*A. pleuropneumoniae* serotype type 5 acapsular mutant)

Typing Isolates

Previously untyped U.S. field isolates of *A. pleuropneumoniae* were assayed by the latex agglutination test and by PCR using Ap1 and Ap5 specific primers (Table 4). Out of 72 field isolates, 58 isolates were typed as *A. pleuropneumoniae* serotypes 1, 5, or 7 by latex agglutination and 14 isolates were determined not to be serotypes 1, 5, or 7. Ten isolates typed as serotype 1 were also identified as serotype 1 by multiplex PCR by the amplification of the 880 bp *cpx* and 1.6 kb *cps* bands. Twenty-two isolates typed as serotype 5 were also determined to be serotype 5 by multiplex PCR by amplification of the 715 bp *cpx* and 1.1 kb *cps* bands. Multiplex PCR using Ap1 and Ap5 specific primers was also performed on 28 isolates typed as serotype 7, and 13 isolates that remained untyped. The 715 bp and 880 bp bands representing the *cpxD* gene was amplified in all strains typed as serotype 7 and identified them as *A. pleuropneumoniae*. The 1.6 kb Ap1-specific and 1.1 kb Ap5-specific bands were not amplified, confirming they were neither serotypes 1or 5. The 715 bp or 880 bp *cpxD* bands were not amplified in the untyped isolates indicating that they were not *A. pleuropneumoniae.* These isolates also grew on blood agar and further confirmed that they were not *A. pleuropneumoniae*. Figure 10 shows amplification of serotype 1 and serotype 5 DNA from field isolates using the multiplex PCR.

Table 4. Comparison of Latex Agglutination and PCR Results from field isolates

	Latex Agglutination Results			PCR Results	
Isolate	Ap1	Ap 5	Ap 7	Ap1	Ap5
Ap 42663-95	+	-	-	+	-
W94 45876-B1	+	-	-	+	-
Ap 5203-96	+	-	-	+	-
41165	+	-	-	+	-
W93 24644	+	-	-	+	-
W94 47301	+	-	-	+	-
41166	+	-	-	+	-
41169	+	-	-	+	-
W95 570	-	+	-	-	+
W93 14691	-	+	-	-	+
W93 18776	-	+	-	-	+
W93 1896	-	+	-	-	+
W93 1294	-	+	-	-	+
App 2020 96	-	+	-	-	+
Ap W93 261	-	+	-	-	+
5091 96	-	+	-	-	+
W93 11859	-	+	-	-	+
W93 10509	-	+	-	-	+
W94 47549	-	+	-	-	+
W93 1912	-	+	-	-	+
28639	-	+	-	-	+
W93 12666	-	+	-	-	+
W93 11175	-	+	-	-	+
W93 43685-D1	-	+	-	-	+
W93 25017	-	+	-	-	+
W93 1030	-	+	-	-	+
W94 47482	-	+	-	-	+
44393 95	-	+	-	-	+
3079 96	-	+	-	-	+
W93 18716	-	+	-	-	+
Ap 42663 95	-	-	+	-	-
W94 47496	-	-	+	-	-
43668 A1	-	-	+	-	-
App 5508 96	-	-	+	-	-
36695 95	-	-	+	-	-

* Isolates received from Rollins Diagnostic Lab, NC

42667 95	-	-	+	-	-
47592 95	-	-	+	-	-
5372 96	-	-	+	-	-
Ap W93 32803	-	-	+	-	-
W94 44372 B2	-	-	+	-	-
1061 96	-	-	+	-	-
W93 12083	-	-	+	-	-
W93 32293	-	-	+	-	-
W93 32510 B1	-	-	+	-	-
W94 45873 A1	-	-	+	-	-
W95 276	-	-	+	-	-
W 93 24644	-	-	+	-	-
W93 15318	-	-	+	-	-
W93 32890	-	-	+	-	-
42882	-	-	+	-	-
W93 32510 A4	-	-	+	-	-
W94 45392 A1	-	-	+	-	-
32098	-	-	+	-	-
39070 95		-	+	-	-
5372 96	-	-	+	-	-
3515 96	-	-	+	-	-
315 96	-	-	+	-	-
4491 96	-	-	+	-	-
W94044372	-	-	-	-	-
W93 30980	-	-	-	-	-
W95 573	-	-	-	-	-
W93 1290	-	-	-	-	-
W93 1287	-	-	-	-	-
W93 1291	-	-	-	-	-
HP W93 30106	-	-	-	-	-
HPS 1518 96	-	-	-	-	-
HPS 15945	-	-	-	-	-
HPS 15206	-	-	-	-	-
HP W93 28398	-	-	-	-	-
5091	-	-	-	-	-
W93	-	-	-	-	-
W94 47297	-	-	-	-	-

* Isolates received from Rollins Diagnostic Lab, NC

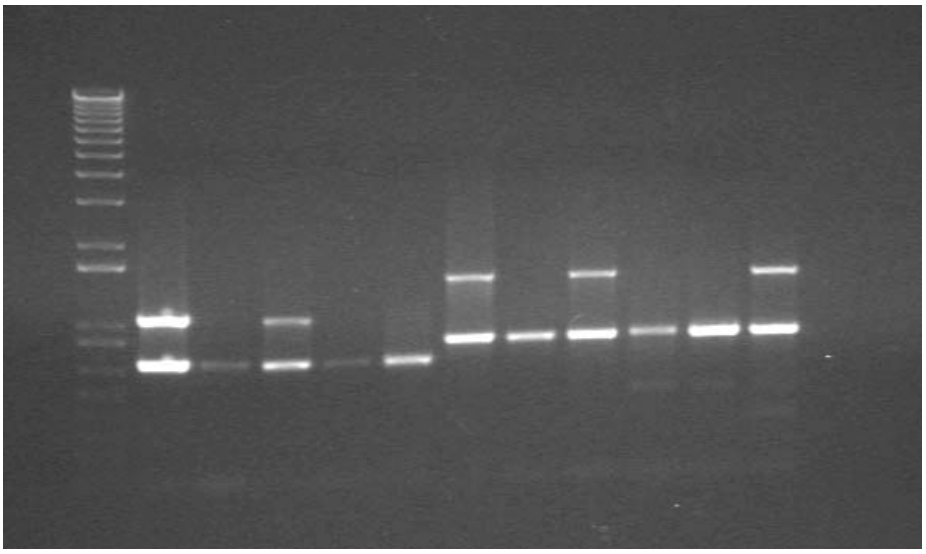

Figure 10. Agarose gel electrophoresis of *A. pleuropneumoniae* field isolates amplified from Ap1 and Ap5 primers. Lane 1, 1-kb ladder, lanes 2-6, field isolates amplified from Ap5A and Ap5B and Ap5C and Ap5D primers, lanes 7-12, field isolates amplified from $Ap1U_1$ and $Ap1L_2$ and $cpxU_1$ and $cpxL_1$ primers.

Due to the prevalence of *A. pleuropneumoniae* serotype 8 in Denmark as compared to the U.S., primers designed specifically for the Ap8 *cps* region were tested at the Danish Veterinary Institute in Copenhagen, Denmark. The primers were used in a PCR assay to screen 38 field isolates, previously determined to be *A. pleuropneumoniae* serotype 8 by gel immunodiffusion and latex agglutination, as well as 2 known *A. pleuropneumoniae* serotype 6 field isolates and the *A. pleuropneumoniae* serotype 6 reference strain Femo. Thirty-three of the 38 *A. pleuropneumoniae* serotype 8 field isolates were confirmed to be *A. pleuropneumoniae* serotype 8 by amplification of the 970 bp fragment. The serotype of the remaining 5 field isolates was not confirmed. The 970 bp fragment was not amplified in the two *A. pleuropneumoniae* serotype 6 field isolates or the *A. pleuropneumoniae* serotype 6 reference strain Femo (Table 5).

Table 5. PCR assay of *A. pleuropneumoniae* reference strains and Danish field isolates using Ap8 U_1 and Ap8 L_1 primers

		PCR product
Strain	# of isolates tested	cps
Ap8 field isolates	38	33+/5-
Ap6 field isolates	2	-
Ap8 reference strain 405	1	+
Ap6 reference strain Femo	1	-

Discussion

All *A. pleuropneumoniae* serotypes are identified based on their unique capsular polysaccharide (Inzana and Mathison, 1987). Cross-reactions frequently occur when serological assays are used for serotyping (Nielsen, 1984; Nicolet, 1988; Mittal et al., 1993). Due to the serotype-specificity of the capsular polysaccharide, it is an ideal target for typing by PCR. Culture methods may only detect *A. pleuropneumoniae* 50% of the time and identification can be difficult due to contaminating microflora (Savoye et al., 2000). PCR is shown to be more sensitive than culture for detection of *A. pleuropneumoniae* (Gram et al., 1996) and the use of serotype-specific primers described here, and by Lo et al. (1998) and Jessing et al. (2001) increases the specificity of the assay. Hennesy et al. (1995) reported the use of an arbitrarily primed PCR assay to serotype *A. pleuropneumoniae,* however this method is highly susceptible to contamination. Various PCR assays have been developed that detect *A. pleuropneumoniae* but the majority of these assays cannot identify the serotype (Moral et al., 1999; Shaller et al., 1999; Chiers et al., 2002). Gram and Ahrens reported the development of two PCR assays that detect *A. pleuropneumoniae* and divide the serotypes into four distinct groups by amplification of an outer membrane protein (Gram and Ahrens, 1998; Gram et al., 2000b). Culture is still needed with these assays to identify the serotype for epidemiological purposes and to monitor the spread of disease. Lo et al. (1998) described the use of a multiplex PCR assay to simultaneously detect *A. pleuropneumoniae* and identify serotype 5. Jessing et al. (2002) reported using primers specific to the capsular polysaccharide regions of *A. pleuropneumoniae* serotypes 2, 5

and 6 and primers to an outer membrane protein in a multiplex PCR assay to simultaneously detect all three serotypes. The current study describes an expansion of the *A. pleuropneumoniae* serotype 5 specific multiplex PCR assay to include the identification of serotypes 1, 2, and 8. The capsular polysaccharide biosynthesis regions of *A. pleuropneumoniae* serotypes 1 and 2 were previously cloned in our lab and sequenced. This thesis research describes the cloning and sequencing of a portion of the *A. pleuropneumoniae* serotype 8 capsular polysaccharide biosynthesis region.

The serotype 8 capsule locus region was located on separately cloned fragments: a 3.7 kb *ClaI* fragment, a 1.6 kb *EcoRV* fragment, and a 2.0 kb *EcoRV* fragment (Figure 1) upstream from the *cpxDCBA* gene cluster involved in *A. pleuropneumoniae* capsular polysaccharide export. The location of the *A. pleuropneumoniae* serotype 8 specific DNA upstream from the *cpxD* gene is consistent with the location of the capsular polysaccharide biosynthesis genes from serotypes 1, 2, 5, (Ward and Inzana, 1997) and other bacterial species that express type II or type III capsules (Russo et al., 1998; Clarke et al., 1999; Lo et al., 2001). These findings provide further evidence that the genetic organization of the *A. pleuropneumoniae* capsule locus is identical between serotypes and very similar to the organization of the type III capsule loci of *H. influenzae* type b, and *N. meningitidis* group B.

The lack of homology observed at the nucleotide level between the ORF's (*cps8A, cps8B, cps8C* and *cps8D*) identified in *A. pleuropneumoniae* serotype 8 and the sequences in the combined nucleotide database at NCBI, was not surprising. A lack of substantial homology at the nucleotide level was also noticed when analyzing genes from the capsular

biosynthesis regions of *A. pleuropneumoniae* serotype 2 (personal communication) and serotype 5 (Ward and Inzana, 1997). These findings reflect the unique structure of the capsular polysaccharide of each serotype. The exception was *cpsB*, which had 82% identity at the nucleotide level with the *tagD* from the techoic acid biosynthesis locus in *B. subtilis*.

A portion of the *A. pleuropneumoniae* serotype 2 *cps* region 3 locus involved in capsular polysaccharide export has been identified and sequenced. Due to the similarity in organization of the capsule gene clusters of *M. haemolytica* and *A. pleuropneumoniae,* a 971 bp fragment of region 3 from *A. pleuropneumoniae* serotype 2 was identified by PCR amplification using primers specific for *M. haemolytica*. Significant homology was observed at the amino acid level between the sequenced fragment of *A. pleuropneumoniae* serotype 2 and sequences in the database, specifically that of *P. multocida.* These findings strongly suggest that not only is the organization of type II and type III capsule gene clusters similar, but that the sequence obtained from the conserved export regions may also be very similar. The 971 bp fragment of the region 3 capsular polysaccharide export region was also identified in serotypes 7 and 8 by PCR and partially sequenced (data not shown). The sequences obtained from these amplified fragments however, showed less homology to *P. multocida* than to *A. pleuropneumoniae* serotype 2.

Both the *cpx* and *cps* regions of *A. pleuropneumoniae* serotypes 1, 2, and 8 were successfully amplified from purified DNA and bacterial colonies. The *cpx* and *cps* regions of serotype 1 were also successfully amplified in lung tissue samples. The use of multiplex

PCR provided the advantage of simultaneous detection of *A. pleuropneumoniae* and identification of serotypes 1, 2, or 8 by use of multiple primer sets.

Primers designed specifically for the *A. pleuropneumoniae* serotype 1 *cps* region amplified a 1.0 kb fragment by PCR (data not shown). However, due to the *A. pleuropneumoniae* serotype 5 multiplex PCR assay, which amplified a 1.1 kb fragment and due to the proximity in size of the *cpx* and *cps* bands amplified in the *A. pleuropneumoniae* serotype 1 multiplex PCR, primers for the *cps* region of *A. pleuropneumoniae* serotype 1 were redesigned to amplify a 1.6 kb fragment from PCR. A longer extension time in the PCR thermocycler was required for amplification of the 1.6 kb *A. pleuropneumoniae* serotype 1 specific fragment. The need for double the concentration of *A. pleuropneumoniae* serotype 1 *cps* primers as compared to *cpx* primers in the PCR reaction, was also important for amplification of the 1.6 kb fragment. This may have been due to competition between the *cps* and *cpx* primers or simply due to the requirement of more *cps* primers to amplify a larger fragment (Innis et el., 1995).

The amplification of the 880 bp cpx product in all serotypes except serotype 4 when using the *cpx* U_1 and *cpx* L_1 primers, demonstrates the conserved nature of the capsular polysaccharide export region among the *A. pleuropneumoniae* serotypes. The absence of the 880 bp fragment in serotype 4 also indicates that areas of non-homology or very low homology may also be present in this conserved region. Similar results were seen when primers to the conserved export region were used in the multiplex PCR assay to identify serotype 5. The export band was amplified in all serotypes except serotype 4 (Lo et al., 1998). Both sets of export primers that did not amplify a fragment in serotype 4 were

designed from the *cpx*C and *cpxD* genes. Primers that did amplify a fragment in serotype 4 were designed from the *cpxA* gene, which encodes for a portion of an ATP binding cassette that is essential for the export of capsule, therefore the relative homology of this gene between different *A. pleuropneumoniae* serotypes should be high. Due to this, it is plausible that primers to the *cpxA* gene would be more likely to amplify this region in all serotypes. The primers designed to the *cpxA* gene within the *cpx* region amplified a 489 bp *cpx* product from all 12 serotypes, including serotype 4. Due to the amplification of the *cpx* region in all 12 serotypes with the *cpxA* primers as compared to the *cpx* U_1 and *cpx* L_1 primers, the new *cpxAF* and *cpxAR* primers were used with the *A. pleuropneumoniae* serotype 8 specific primers. Due to previous optimization of the *cpx* U_1 and *cpx* L_1 primers with *A. pleuropneumoniae* serotypes 1 and 2, these multiplex PCR primer sets would continue to be used together. The $MgCl_2$ concentration was the most important parameter for the specificity of each of the serotype-specific PCR reactions and was used to control the amplification of non-specific fragments.

The successful application of the multiplex PCR assay to field isolates provides a quick and simple method of identifying *A. pleuropneumoniae* serotypes 1, 2, 5 and 8. The preparation and identification of the isolates was relatively simple and the PCR amplification occurred in less than 4 hours. The amplification of the 880 bp and 1.6 kb fragments from the *A. pleuropneumoniae* serotype 1 multiplex PCR and the 750 bp and 1.1 kb fragments from the *A. pleuropneumoniae* serotype 5 multiplex PCR, were not inhibited by the presence of other respiratory organisms. Previous optimization of the *A. pleuopneumoniae* serotype 1 and serotype 5 specific PCR's allowed increased specificity

in detecting only *A. pleuropneumoniae*. However, the specificity of the PCR reactions can be affected by using too much template, specifically when using bacterial cells as seen in Figure 9. The non-Ap isolates do slightly amplify fragments that are similar in size to the Ap specific fragments, but due to the intensity of the Ap specific fragments, the two can be distinguished. The use of this multiplex PCR assay eliminated cross-reactions typically observed with serological detection assays (Nicolet, 1988; Nielsen and O'Connor, 1988). The *A. pleuropneumoniae* serotype 8 specific primers were successfully used to confirm the identity of 33 serotype 8 field isolates previously determined to be serotype 8. Cross-reactivity between serotypes 6 and 8 has been problematic, but no cross-reactions were seen with the limited number of serotype 6 isolates tested.

The potential for using this assay with clinical specimens was also investigated. *A. pleuropneumoniae* serotype 1 was successfully amplified from lung tissue samples of infected swine. The use of *A. pleuropneumoniae* serotype 1 *cps* primers $Ap1U_1$ and $Ap1L_1$ amplified a 1.0 kb fragment, amplified the A *pleuropneumoniae* serotype 1 DNA with greater sensitivity than the *cps* primers $Ap1U_1$ and $Ap1L_2$, which amplified a 1.6 kb fragment. The amplification of the larger 1.6 kb fragment may have been difficult due to inhibitors that may be found in lung tissue samples. The $Ap1U_1$ and $Ap1L_1$ *cps* primers successfully amplified *A. pleuropneumoniae* DNA in all lung tissue samples tested. This multiplex PCR assay has proven to be faster and more sensitive than routine culture to identify and serotype *A. pleuropneumoniae*. The use of primers specific to *cps* regions of

other serotypes will expand the assay and result in the detection and identification of all 12 serotypes.

In conclusion, the multiplex PCR assay described was effective in detecting *A. pleuropneumoniae* and identifying serotypes 1, 2, and 8 from purified DNA and bacterial cells. The *A. pleuropneumoniae* serotype 1 multiplex PCR was able to detect *A. pleuropneumoniae* and identify serotype 1 from lung tissue samples. This assay is very quick and easy to perform and is highly sensitive and specific compared to serological assays. This assay can be applied to all 12 serotypes of *A. pleuropneumoniae* once serotype-specific DNA sequences are determined.

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Vita

Jennifer Schuchert was born on January 6, 1972. She grew up in Richmond, Virginia and has one sister. Her father is a mechanical engineer and her mother is the director of Prevent Child Abuse Virginia. Jennifer received her undergraduate degree in Biology at Randolph-Macon College in Ashland, Virginia in December 1994. From 1994 –1998 she worked in a pediatricians office as a medical assistant in Richmond. In 1996 Jennifer met her husband in Richmond and in 1997 her husband transferred from a local community college to Virginia Tech to pursue a degree in engineering. A year later Jennifer followed him to Virginia Tech. In 1999 Jennifer was accepted to the graduate school at the Virginia-Maryland Regional College of Veterinary Medicine under the supervision of Dr. Thomas Inzana, where she conducted research on *Actinobacillus pleuropneumoniae*. Jennifer will move to Fairfax, Virginia after her defense to begin a job at the American Type Culture Collection and to be with her husband, whom she wed just 7 months ago.