

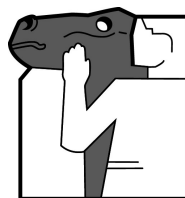


**A PATHOGENETIC APPROACH TO VACCINATION AGAINST
PLEUROPNEUMONIA IN SWINE**

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CONTENTS

List of abbreviations	7
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INTRODUCTION

CONTAGIOUS PORCINE PLEUROPNEUMONIA: A REVIEW WITH EMPHASIS ON PATHOGENESIS AND DISEASE CONTROL

1. Etiology	11
2. Prevalence and epizootiology	11
3. Clinical signs and lesions	12
4. Pathogenesis	14
5. Role of virulence factors in pathogenesis and protection	15
6. Disease control with emphasis on vaccination	21
7. References	26

SCIENTIFIC AIMS	35
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EXPERIMENTAL STUDIES

CHAPTER 1 EVALUATION OF THE EFFICACY OF COMMERCIALLY AVAILABLE VACCINES AGAINST PLEUROPNEUMONIA

Effects of endobronchial challenge with *Actinobacillus pleuropneumoniae* serotype 9 of pigs vaccinated with inactivated vaccines containing the Apx toxins

	41
Summary	42
Introduction	43
Materials and methods	43
Results	45
Discussion	50
References	51

Effects of endobronchial challenge with *Actinobacillus pleuropneumoniae* serotype 9 of pigs vaccinated with a vaccine containing Apx toxins and transferrin-binding proteins

	53
Summary	54
Introduction	55
Materials and methods	55
Results	57
Discussion	60
References	62

CHAPTER 2	ADHESION OF <i>ACTINOBACILLUS PLEUROPNEUMONIAE</i> TO PORCINE ALVEOLAR EPITHELIAL CELLS <i>IN VITRO</i> AND <i>IN VIVO</i>	
	Characterization of the <i>in vitro</i> adhesion of <i>Actinobacillus pleuropneumoniae</i> to alveolar epithelial cells	67
	Summary	68
	Introduction	69
	Materials and methods	69
	Results	73
	Discussion	81
	References	85
	Effect of culture conditions of <i>Actinobacillus pleuropneumoniae</i> serotype 2 and 9 strains on <i>in vivo</i> adhesion to alveoli of pigs	89
	Summary	90
	Introduction	91
	Materials and methods	91
	Results	93
	Discussion	93
	References	95
CHAPTER 3	EVALUATION OF THE EFFICACY OF A VACCINE CONTAINING CANDIDATE-ADHESINS	
	Effect of endobronchial challenge with <i>Actinobacillus pleuropneumoniae</i> serotype 10 of pigs vaccinated with bacterins consisting of <i>Actinobacillus pleuropneumoniae</i> serotype 10 grown under NAD-rich and NAD-restricted conditions	99
	Summary	100
	Introduction	101
	Materials and methods	102
	Results	105
	Discussion	108
	References	110
	GENERAL DISCUSSION	113
	SUMMARY	129
	SAMENVATTING	133

DANKWOORD	137
CURRICULUM VITAE	141

LIST OF ABBREVIATIONS

™: trade mark

μl: microliter

Apx: *Actinobacillus pleuropneumoniae* exotoxin

PBSS: phosphate buffered salt solution

kDa: kiloDalton

cfu: colony forming units

mg: milligram

mm: millimeter

NAD: nicotinamide-adenine dinucleotide

nm: nanometer

OD: optical density

RTX: Repeat in ToXins

SDS-PAGE: sodiumdodecylsulphate polyacrilamide gel electrophoresis

SPF: specific pathogen free

UV: ultra violet light

CONTAGIOUS PORCINE PLEUROPNEUMONIA: A REVIEW WITH EMPHASIS ON PATHOGENESIS AND DISEASE CONTROL

- 1. ETIOLOGY**
- 2. PREVALENCE AND EPIZOOTIOLOGY**
- 3. CLINICAL SIGNS AND LESIONS**
- 4. PATHOGENESIS**
- 5. ROLE OF VIRULENCE FACTORS IN PATHOGENESIS AND
PROTECTION**
- 6. DISEASE CONTROL WITH EMPHASIS ON VACCINATION**
- 7. REFERENCES**

1. ETIOLOGY

Actinobacillus pleuropneumoniae (*A. pleuropneumoniae*) is an obligate parasite of the porcine respiratory tract (Taylor, 1999). The bacterium is a small, Gram-negative capsulated rod with typical coccobacillary morphology (Nicolet, 1992). Based on nicotinamide adenine dinucleotide (NAD) requirements, *A. pleuropneumoniae* can be divided into 2 biotypes. Biotype 1 strains are NAD-dependent whereas biotype 2 strains are NAD-independent. So far, 15 serotypes have been described (Blackall et al., 2002) although serotypes 1 and 5 are subdivided into 1a and 1b and 5a and 5b, respectively (Jolie et al., 1994; Nielsen, 1986; Nielsen et al., 1997). All serotypes are haemolytic and produce a positive CAMP (Christie, Atkins, Munch-Peterson) reaction with beta-haemolytic *Staphylococcus aureus* (Taylor, 1999). The incomplete haemolysis zone induced by the β -toxin is converted in a complete zone of haemolysis around the *A. pleuropneumoniae* colony. Four toxins are produced: ApxI, II, III and IV (Dom et al., 1994a; Frey et al., 1993; Frey et al., 1994; Jansen, 1994; Kamp et al., 1991; Schaller et al., 1999). Serotyping is mainly based on capsular antigens. Furthermore, the serotypes have different lipopolysaccharide (LPS) composition, except that serotypes 1, 9 and 11, serotypes 3, 6 and 8 and serotypes 4 and 7 have common epitopes. Although there is evidence that all serotypes of *A. pleuropneumoniae* can cause severe disease and death in pigs, significant differences in virulence have been observed (Frey, 1995; Rogers et al., 1990; Rosendal et al., 1985). These variations may be partly attributed to the production of different combinations of Apx toxins, with the most virulent serotypes producing both Apx I and Apx II (Frey, 1995). Field observations and experimental infections provide evidence that biotype 2 strains are less virulent than biotype 1 strains. Field observations also indicate that biotype 1 serotype 1a, 1b, 5a, 5b, 9 and 10 strains are more virulent than the other biotype 1 serotypes. This was, however, not confirmed under experimental conditions (Dom and Haesebrouck, 1992a; Jacobson et al., 1995).

2. PREVALENCE AND EPIZOOTIOLOGY

Pleuropneumonia is a major problem in much of Europe, the USA, Canada and Eastern Asia. Control measures may suppress clinical disease but reports from many countries suggest that 30-50% of all pigs are infected. In Belgium, the biotype 1-serotypes 2, 3, 5, 6, 7, 8, 9 and 11 strains and the biotype 2-serotype 2 strains are mostly found (Hommez et al., 1988; Hommez et al., 1990).

A. pleuropneumoniae can be isolated from nasal cavities, tonsils, middle ear cavities and lungs of infected pigs (Dom et al., 1994; Duff et al., 1996; Sidibe et al., 1993). The bacterium is normally not considered as invasive, but there is one report of *A. pleuropneumoniae* being recovered from osteomyelitis in pigs (Jensen et al., 1999). The bacterium is mainly transmitted by direct contact between infected pigs or by aerosols. After clinical or subclinical infections, pigs can become carriers of *A. pleuropneumoniae*. In such pigs, the infectious

agent is located mainly in necrotic lung lesions and/or tonsils, less frequently in the nasal cavities (Nicolet, 1992; Sidibé et al., 1993).

Transmission between herds occurs through the introduction of carriers to populations without previous experience of the disease. *A. pleuropneumoniae* is a strict pathogen of the porcine respiratory system, has a very short survival time in the environment and is very fragile and sensitive to the usual disinfectants (Taylor, 1999). The bacterium can survive for a few days in mucus or other organic material (Nicolet, 1992). In case of acute outbreaks of pleuropneumonia, indirect transmission can occur via exudate on booths or clothing (Nicolet, 1992).

An increased incidence of pleuropneumonia is associated with stress situations such as transports, stable changing, overcrowding and inappropriate housing (Nicolet, 1992). Another trigger factor is infection with other respiratory pathogens. It was demonstrated that a concomitant infection with *Mycoplasma hyopneumoniae* (Caruso and Ross, 1990; Yagihashi et al., 1984) or with Aujeszky's disease virus (Sakano et al., 1993) can worsen the symptoms of pleuropneumonia. In contrast, a concomitant experimental infection with PRRSV had no effect on clinical symptoms and lesions caused by *A. pleuropneumoniae* (Pol et al., 1997).

Sows from a chronically infected herd confer passive immunity to their offspring through colostral antibodies (Nielsen, 1985). As the colostral antibody level declines, the piglets become susceptible to infection. Where the infection is enzootic, the condition is mostly found amongst pigs of 6-12 weeks of age.

3. CLINICAL SIGNS AND LESIONS

The pace of disease can range from peracute to chronic depending on the serotype, the immune status of the host, and the infection doses (Crujisen et al., 1995; Hensel et al., 1993; Rogers et al., 1990; Rosendal et al., 1985; Sebunya et al., 1983). Peracutely or acutely diseased pigs may have some or all of the following clinical symptoms: high fever, increased respiratory rate, coughing, sneezing, dyspnoea, anorexia, ataxia, vomiting, diarrhoea and severe respiratory distress with cyanosis and presence of haemorrhagic foam on mouth and/or nostrils (Ajito et al., 1996; Ligget et al., 1987; Rosendal et al., 1985; Taylor, 1999).

The subacute and chronic forms develop after the disappearance of acute signs. Recovering animals may cough, and show respiratory distress particularly when disturbed. Exercise intolerance may continue for days and affected animals may have reduced appetite, appear gaunt and hairy, be depressed and show reduced rates of liveweight gain.

Lesions are mainly characterised by a hemorrhagic necrotizing pneumonia and fibrinous pleuritis (Figure 1). The pneumonia is mostly bilateral, with involvement of the cardiac and apical lobes, as well as at least part of the diaphragmatic lobes where pneumonic lesions are often focal and well demarcated. In the peracute and acute form of the disease, pulmonary lesions are characterised by severe oedema, inflammation, haemorrhage and necrosis (Ajito et al., 1996; Bertram et al., 1985; Rosendal et al., 1985). The thoracic cavity is often filled with bloody fluid and fibrin clots. Diffuse fibrinous pleuritis and pericarditis are also common

(Rosendal et al, 1985). Tracheobronchial and mesenteric lymph nodes can have oedema and become swollen as a result of neutrophil infiltration and fibrin deposition (Ajito et al., 1996; Ligget et al., 1987; Rosendal et al., 1985). Animals that survive infection may have complete resolution of lesions, but frequently they retain necrotic foci, encapsulated abscesses and/or adhesive pleuritis (Ligget et al., 1987; Rosendal et al., 1985) (Figure 2).

Histologically, in the early stages of disease, polymorphonuclear leukocyte (PMN) infiltration, oedema and fibrinous exudate are present (Ajito et al., 1996; Bertram et al., 1985; Ligget et al., 1987). In the later stages, macrophage infiltration is more apparent and necrotic areas are surrounded with dense bands of degenerating leukocytes (Ajito et al., 1996; Bertram et al., 1985; Ligget et al., 1987). Within alveoli, degeneration of pulmonary epithelial cells, macrophages and PMNs is seen (Ajito et al., 1996; Perfumo et al., 1983). Severe necrotising vasculitis leads to a disrupted blood-lung barrier resulting in haemorrhage (Ligget et al., 1987; Rosendal et al., 1985; Serebrin et al., 1991). Degenerating erythrocytes, fibrin and platelet thrombi are found within dilated capillaries in the lung (Perfumo et al., 1983).

The bacteria can be found within the alveolar and interlobular fluid and they may spread via lymph vessels from the parenchyma to the pleura, but bacteraemia is rare (Ajito et al., 1996). Large numbers of bacteria are phagocytosed by macrophages and PMNs. The bacterium does not invade epithelial cells (Min et al., 1998).



Figure 1. Hemorrhagic necrotizing pneumonia (left) and fibrinous pleuritis (right) in acute *A. pleuropneumoniae* infections

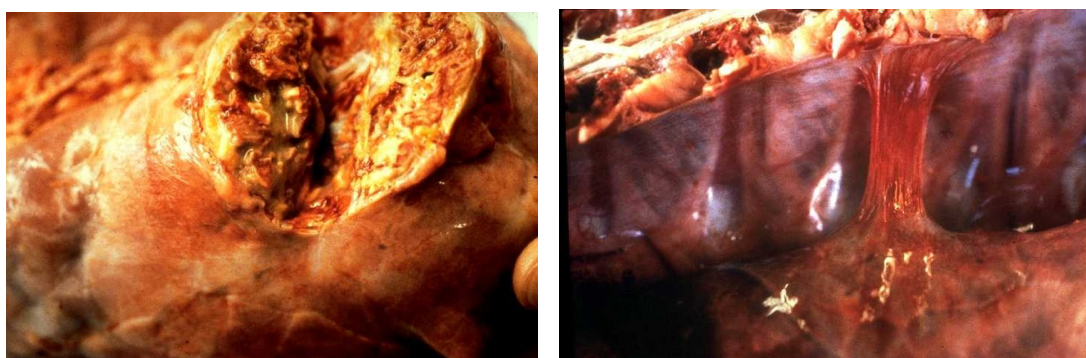


Figure 2. Abscess (left) and adhesive pleuritis (right) in chronic *A. pleuropneumoniae* infections

4. PATHOGENESIS

The pathogenesis of porcine pleuropneumonia is considered to be multifactorial (Nicolet, 1992). There are three basic stages in the pathogenesis: colonisation, evasion of host clearance mechanisms, and damage to host tissues.

Colonisation

Colonisation, the ability of a pathogen to adhere to host cells or surfaces and to multiply within the host, is generally regarded as an important prerequisite for virulence manifestation of bacteria (Ofek and Beachy, 1980). It was demonstrated that *A. pleuropneumoniae* does not bind well to the cilia or epithelium of the trachea or bronchi but does bind intimately with the cilia of terminal bronchioli and epithelial cells of the alveoli (Dom et al., 1994). Thus, while *A. pleuropneumoniae* can be isolated from the tonsils and nasal cavities of pigs (Chiers et al., 1999; Sidibe et al., 1993) it is not yet clear if colonisation of the upper respiratory tract is necessary for pulmonary infection in naturally occurring cases of pleuropneumonia. This may depend on the nature of the infectious material encountered by the animal (aerosol or mucus secretions). Aerosol particles are small enough to penetrate into the lower respiratory tract, obviating the need for colonisation of the upper respiratory tract (Kaltrieder et al., 1976).

Evasion of host clearance mechanisms

Rapid clearance of bacteria from the respiratory tract is an effective host defence against bacterial infections in the lung. A number of defence mechanisms clear or destroy any bacteria inhaled with air or fortuitously deposited in the airway passages. Nasal clearance is the removal of particles, including aerosols carrying micro-organisms that are deposited near the front of the airway. Those deposited on the nonciliated epithelium are normally removed by sneezing or blowing, whereas those deposited posteriorly are swept over the mucus-lined ciliated epithelium to the nasopharynx, where they are swallowed. Tracheobronchial clearance is accomplished by mucociliary action: the beating motion of cilia moves mucus continuously from the lung toward the oropharynx. Particles deposited on this film are eventually either swallowed or expectorated. In the alveoli, bacteria can be eliminated by the action of phagocytic cells. In healthy animals, macrophages are the predominant phagocyte found in the lower respiratory tract, whereas the number of PMNs is generally small, but increases rapidly following infection (Bertram et al., 1985; Sibille et al., 1990). Alveolar macrophages (AMs) are strategically situated at the air-surface interface in the alveoli, and are thus the first cells to encounter inhaled organisms. Both macrophages and PMNs phagocytose *A. pleuropneumoniae*. Following phagocytosis, PMNs can effectively kill *A. pleuropneumoniae* whereas macrophages cannot (Crujisen et al., 1992). This is probably due to the more potent bactericidal capacity of PMNs (Crujisen et al., 1992; Sibille et al., 1990). *A. pleuropneumoniae* may survive for more than 90 minutes within macrophages, during which time liberation of Apx toxins may result in lysis of these phagocytes (Crujisen et al., 1992). These Apx toxins are the major factors involved in the impairment of phagocytic

function of macrophages and PMNs. Furthermore, *A. pleuropneumoniae* produces several factors which may contribute to its ability to survive within the macrophages: capsule and lipopolysaccharides (Bilinski et al., 1991); copper-zinc superoxide dismutase (Langford et al., 1996); stress proteins (Fuller et al., 2000); and ammonia (Bossé et al., 2000).

Damage to host tissues

Most of the pathological consequences of pleuropneumonia can be attributed to the Apx toxins which exert cytotoxic effects on endothelial cells (Serebrin et al., 1991), macrophages (Dom et al., 1992b), neutrophils (Dom et al., 1992a) and alveolar epithelial cells (Van de Kerkhof et al., 1996). Activation of neutrophils, alveolar and intravascular macrophages, largely due to Apx toxins and LPS, leads to release of toxic oxygen metabolites, as well as proteolytic enzymes and various cytokines (Dom et al., 1992a; Dom et al., 1992b; Sibille et al., 1990; Pabst, 1996; Udeze et al., 1987). LPS can enhance the effects of Apx toxins on phagocytes (Fenwick, 1994).

5. ROLE OF VIRULENCE FACTORS IN PATHOGENESIS AND PROTECTION

Different virulence factors have been described, including capsules, lipopolysaccharides, outer membrane proteins, transferrin binding proteins, proteases, Apx toxins and adhesins (Figure 3).

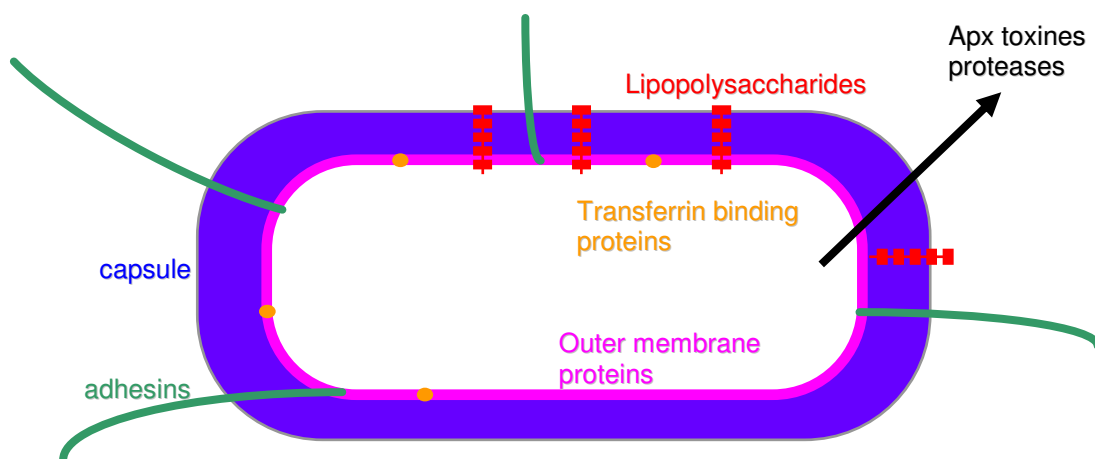


Figure 3. Virulence factors of *A. pleuropneumoniae*.

Capsule

Capsules are found in all strains of *A. pleuropneumoniae*. They mainly consist of derivatized repeating oligosaccharides that determine serotype specificity (Beybon et al., 1993; Perry et al., 1990). The capsule is responsible for the characteristic iridescence of the colony on a clear medium.

The chemical composition and structure of the capsule for the serotypes 1-12 have been determined (Perry et al., 1990). In general, these consist of repeating oligosaccharide units (serotypes 5a, 5b and 10), teichoic acid polymers joined by phosphate diester bonds (serotypes 2, 3, 6, 7, 8, 9 and 11) or oligosaccharide polymers joined through phosphate bonds (serotypes 1, 4 and 12) (Perry et al., 1990).

The DNA region involved in export of the capsular polysaccharide of *A. pleuropneumoniae* serotype 5a has been identified and characterized (Ward and Inzana, 1996).

Variation in virulence can be attributed, at least in part, to the composition and structure of the capsule or the amount of capsular polysaccharides on the cell. Using electron microscopy, a direct correlation between the virulence of the strain and the thickness of the capsule was demonstrated (Jensen and Bertram, 1986).

Although purified *A. pleuropneumoniae* capsular polysaccharides do not induce clinical illness or lesions in pigs (Fenwick et al., 1986), the capsule is essential for *A. pleuropneumoniae* virulence *in vivo* (Tascon et al., 1996), probably as a virulence factor that allows the bacterium to resist the antibacterial environment produced by the host's immune system. The capsular polysaccharides protect *A. pleuropneumoniae* against phagocytosis and lysis by complement (Inzana et al., 1988).

Antibodies directed against the capsule opsonize the bacterium and may play a role in serotype specific partial protection induced by vaccination with bacterins. Inzana et al. (1991, 1993) showed that a non-encapsulated Apx toxin producing mutant gave good protective immunity against *A. pleuropneumoniae* challenge while a non-toxin producing but capsulated mutant gave virtually no protection. This demonstrates that Apx toxins are more important in protection than capsule.

Lipopolysaccharides

Lipopolysaccharides (LPS) are essential structural components of the outer membrane of Gram-negative bacteria. They consist of a polysaccharide and a lipid A moiety, of which the latter is a toxic compound (endotoxin). The polysaccharide moiety consists of a core and O side chains. This typical complete structure is referred to as the smooth (or S-form) chemotype. Strains which have lost the O-polysaccharides are referred to as the rough (or R-form) chemotype. An intermediate form (with one or a limited number of O-side chains), called semi-rough, also exists in *A. pleuropneumoniae*.

Even though a capsule is present at the surface of this bacterium, studies have revealed that LPS can traverse the thick capsular material and reach the outmost region of the cell (Paradis et al., 1996).

Although many of the pathological consequences of *A. pleuropneumoniae* infection have been attributed to LPS, extremely large doses of purified LPS are required to induce lesions similar to those found in naturally infected pigs (Fenwick et al., 1986; Udeze et al., 1987). Furthermore, pigs infected with a mutant of a serotype 1 strain lacking Apx toxins, but with

normal LPS, do not develop clinical disease or lung lesions (Tascon et al., 1994). This indicates that LPS is not responsible for the typical *A. pleuropneumoniae* lesions.

LPS activate the alternative complement cascade resulting in release of complement components that attract and activate PMNs and macrophages and stimulate release of inflammatory mediators, resulting in further PMN and platelet activation, vasodilation and constriction of pulmonary airways (Bertram, 1988; Udeze et al., 1987).

LPS have also been implicated in adhesion of *A. pleuropneumoniae* to tracheal mucus, tracheal and lung frozen sections (Paradis et al, 1994) and host glycosphingolipids (Abul-Mihl et al., 1999).

Pigs immunised with LPS were only partially protected against challenge with the homologous *A. pleuropneumoniae* serotype (Inzana, 1988), indicating that LPS may play a role in the partial serotype specific protection that is induced by vaccination with bacterins.

Outer membrane proteins

Several proteins of the outer membrane of *A. pleuropneumoniae* are recognized by convalescent sera. Furthermore, specific outer membrane proteins can be induced under conditions of iron restriction or addition of maltose (Deneer and Potter, 1989a&b; Jansen, 1994). Although outer membrane protein profiles differ for most serotypes of *A. pleuropneumoniae* (Rapp et al, 1986), it has been shown that isolates of all serotypes contain several common outer membrane proteins, including the peptidoglycan-associated lipoprotein PalA of 14 kDa (Frey et al., 1996), a 29/41-kDa heat-modifiable protein, a major protein that varies from 32 to 42 kDa depending on the serotype and a 48-kDa protein (Cruz et al., 1996). DNA sequence analysis of the gene encoding PalA revealed high similarity of the protein's amino acid sequence to that of the *E. coli* peptidoglycan-associated lipoprotein PAL, to the *Haemophilus influenzae* outer membrane protein P6 and to related proteins of several Gram-negative bacteria. This gene is conserved and expressed in all *A. pleuropneumoniae* serotypes and in *A. lignieresii*. A very similar gene is present in *A. suis* and *A. equuli* (Frey et al., 1996).

PalA as well as proteins of 32K and 42K are immunodominant (Frey et al., 1996; Jansen, 1994; MacInnes and Rosendal, 1987). Immunisation with an outer membrane extract or a crude outer membrane preparation conferred limited protection against challenge with *A. pleuropneumoniae* (Beaudet et al, 1994; Jansen, 1994). Immunisation with recombinant outer membrane lipoprotein Oml A (40 kDa), which is probably present in all serotypes of *A. pleuropneumoniae*, protected pigs from death upon challenge with the homologous strain, but lesions were found in the lungs and *A. pleuropneumoniae* was isolated from the lungs (Gerlach et al, 1993). This indicates that antibodies against Oml A may contribute to, but are not sufficient for protection of pigs against *A. pleuropneumoniae* infection (Jansen, 1994).

Transferrin binding proteins

Iron is essential for bacterial growth. However, it is not readily available in the extracellular environment of the host due to complexation by the host glycoproteins, transferrin and lactoferrin. *A. pleuropneumoniae* expresses a number of factors that are involved in the acquisition and uptake of iron. It is capable of utilising porcine transferrin, but not transferrin from other animal species, as a sole source of iron (Niven et al., 1989). These receptors are expressed under iron limited conditions. They consist of two distinct proteins. Transferrin binding protein A (also known as Tbp1) has an approximate molecular mass of 100 kDa and likely forms a transmembrane channel for transport of iron across the outer membrane (Gonzalez et al., 1995; Wilke et al., 1997). The transferrin binding protein B (also known as Tbp2) has an approximate molecular mass of 60 kDa and appears to be a lipoprotein anchored to the outer membrane by N-terminal fatty acid residues (Fuller et al., 1998; Gerlach et al., 1992; Gonzalez et al., 1995). The pathway of iron acquisition suggested by Kirby et al. (1995) involves binding and iron removal from transferrin at the bacterial surface by the co-ordinate action of TbpA and TbpB followed by transport of iron across the outer membrane via Tbp A and binding of iron by a periplasmic binding protein.

Although both proteins are surface accessible and bind to the C-lobe of porcine transferrin, there is evidence that an interaction between these proteins is required for optimal utilisation of transferrin as a source of iron (Fuller et al., 1998; Gonzalez et al., 1995; Litt et al., 2000). In addition, Gerlach et al. (1992) showed that TbpB is also capable to bind haemin but not haemoglobin. This binding specificity has not been tested for TbpA.

Immunisation of pigs with the TbpB conferred limited protection against challenge with the homologous strains (Gerlach et al., 1992 ; Rossi-Campos et al., 1992). This indicates that Tbp proteins contribute to, but are not sufficient for protection of pigs against *A. pleuropneumoniae* infection (Jansen, 1994).

A. pleuropneumoniae can also use haem compounds including free haem, haemin, haematin and haemoglobin as a source of iron (Deneer and Potter, 1989). All serotypes of *A. pleuropneumoniae* are capable of obtaining haem products via production of haemolysins (Frey et al., 1993).

Proteases

All serotypes of *A. pleuropneumoniae* appear to secrete a high-molecular-mass protease complex (>200 kDa) that degrades porcine gelatine, Ig A and haemoglobin (Garcia-Cuellar et al., 2000; Negrete-Abascal et al., 1994). It has been suggested that the Ig A cleaving proteases could facilitate the mucosal spread of *A. pleuropneumoniae* and that proteolytic cleavage of haemoglobin could be a mechanism of iron acquisition. The exact role of these proteases in the development of pathology has yet to be investigated.

Apx toxins

Four Apx toxins have been described: two hemolytic exotoxins (ApxI and Apx II), one non-hemolytic exotoxin (ApxIII) and one exotoxin that is only produced *in vivo* (ApxIV) (Dom et al., 1994a ; Frey et al., 1993 ; Frey et al., 1994 ; Jansen, 1994; Kamp et al., 1991 ; Schaller et al., 1999). They are toxic for porcine alveolar macrophages and neutrophils and belong to the family of pore forming RTX-toxins, a group of protein toxins which is widely spread among pathogenic Gram-negative bacteria (Frey et al, 1994).

ApxI is a strongly hemolytic and cytolytic protein with an apparent molecular mass of 105 kDa. It was previously named haemolysin I or cytolysin I and shows strong similarities to the *Escherichia coli* α -haemolysin and to a lesser extent to the *Mannheimia haemolytica* leukotoxin. It is produced by serotypes 1, 5, 9, 10 and 11 as well as by *Actinobacillus suis* (Kamp et al., 1994).

ApxII is a weakly hemolytic and weakly cytotoxic exotoxin which was previously named haemolysin II or cytolysin II. It has an apparent molecular mass of 103 kDa and is produced by all serotypes, except serotype 10. It is also produced by *Actinobacillus suis* (Kamp et al., 1994). Based on DNA derived amino acid sequence ApxII is closely related to the *Mannheimia haemolytica* leukotoxin.

ApxIII is not hemolytic but strongly cytotoxic. It was previously named pleurotoxin. It is produced by biotype1 serotype 2, 3, 4, 6 and 8 strains and has an apparent molecular mass of 120 kDa.

In contrast to ApxI, ApxII and ApxIII cytotoxins, each of which is produced by some but not all serotypes, the ApxIV toxin is produced by all serotypes (Cho and Chae, 2001; Schaller et al., 1999). The *apxIV* gene product could not be detected in *A. pleuropneumoniae* cultures grown under various conditions *in vitro*. Pigs experimentally infected with serotypes 1, 5 or 7 produced antibodies that reacted with the ApxIV toxin (Schaller et al., 1999), suggesting that the *apxIV* gene product is induced *in vivo*. The *apxIV* gene was detected in degenerating neutrophils and macrophages by Cho and Chae (2001). This suggests that ApxIV toxin may lead to host-mediated tissue damage by the lysis of inflammatory cells. Further studies on function and regulation of the *apxIV* gene *in vivo* are needed.

The structural toxin genes as well as the genes encoding activation and secretion of the Apx toxins have been characterised (for a review, see Frey et al., 1994; Frey, 1995; Jansen, 1994).

It is now clear that Apx toxins play an important role in the pathogenesis of porcine pleuropneumonia. They play a role in evasion of the hosts first line defence: at high concentrations RTX toxins form pores in membranes of phagocytic and other target cells, resulting in osmotic swelling and cell death. Most of the pathological consequences of porcine pleuropneumonia can be attributed to the Apx I, II and III toxins: they are toxic for endothelial cells (Serebrin et al., 1991) and provoke an oxidative burst in macrophages (Dom et al., 1992c) and neutrophils (Dom et al., 1992b) resulting in excessive production of oxygen radicals which can have deleterious effects on host cells. Moreover, purified recombinant Apx

toxins were able to cause lesions upon endobronchial instillation and mutant strains which are unable to produce Apx toxins did not induce lesions (Kamp et al., 1993; Stockhofe-Zurwieden et al., 1996). Use of transposon mutagenesis (Tascon et al., 1994) and complementation experiments (Reimer et al., 1995) also prove that Apx toxins are essential in the pathogenesis of porcine pleuropneumonia.

It was demonstrated that *A. pleuropneumoniae* and its metabolites are able to kill type II alveolar epithelial cells and that cytotoxicity is at least in part due to production of Apx toxins (Van De Kerkhof et al., 1996). It is remarkable that rabbit sera against ApxI and ApxII were not able to protect alveolar epithelial cells against the biotype 1 serotype 1 strain although such sera were able to neutralize toxicity of culture supernatant. It has been shown that *A. pleuropneumoniae* adheres to alveolar epithelial cells *in vivo* (see below). It was demonstrated that this also occurs *in vitro* (Haesebrouck et al., 1996). This association may mediate delivery of high concentrations of Apx toxins directly to the surface of the alveolar epithelial cells, resulting in destruction of the target cells even in the presence of neutralizing antibodies. It has been described that toxins produced by adhering bacteria are targeted more efficiently and become relatively inaccessible to neutralization by toxin inhibitors (Ofek et al., 1990).

The importance of Apx toxins in protective immunity against porcine pleuropneumonia was demonstrated by immunisation with Apx toxins in combination with other bacterial compounds. In these experiments the Apx toxins were essential vaccine components to confer protection against challenge (Beaudet et al., 1994 ; Byrd and Kadis, 1992 ; Frey, 1995; Van den Bosch et al., 1992 ; Jansen, 1994). However, it has also become clear that the Apx toxins are not the only factors involved in protective immunity.

Adhesins

During the course of many infectious diseases, including pulmonary infections, bacteria colonize body sites by engaging their surface-bound adhesins with cognate receptors available on host cells. The recognition and attachment processes are therefore considered to be the first steps in establishing bacteria at a given site. It is a prerequisite for colonization and virulence manifestation of bacteria (Ofek and Beachy, 1980). Adherence enables colonization to occur and allows the bacterium to exert its pathogenic effects.

It was shown that *A. pleuropneumoniae* adheres to the epithelium of the alveoli or the cilia of the terminal bronchioli of experimentally infected pigs (Dom et al., 1994). It has also been shown that *A. pleuropneumoniae* adheres to porcine tracheal rings maintained in culture (Bélanger et al., 1990), to porcine frozen tracheal and lung sections (Jacques et al., 1991), to erythrocytes from various animal species (Jacques et al., 1988), to tonsillar epithelial cells (Chiers et al., 1999), to swine buccal epithelial cells (Hamer et al., 1999) and to type III swine-lung collagen (Enriquez et al., 1999).

Several surface structures have been described in the family of *Pasteurellaceae* that are involved in adhesion, including the capsule (Confer et al., 1995), fimbriae (Read et al., 1992),

lipopolysaccharides (Bélanger et al., 1990) and outer membrane proteins (Confer et al., 1995). Concerning *A. pleuropneumoniae*, only few adhesion factors have been described. Lipopolysaccharides seem to be involved in the *in vitro* adhesion to porcine tracheal rings (Bélanger et al., 1990) and mucus (Bélanger et al., 1992&1994). It was shown that the capsule is not responsible for adherence to tracheal frozen sections (Jacques, 1999). Type 4 fimbriae have been demonstrated on *A. pleuropneumoniae* (Dom et al., 1994a; Utrera and Pijoan, 1991; Zhang et al. 2000) but their role in adherence needs to be elucidated.

6. DISEASE CONTROL WITH EMPHASIS ON VACCINATION

Disease control of pleuropneumonia may be accomplished in a number of different ways: (1) antimicrobial treatment, (2) vaccination, (3) isolation-treatment-vaccination combination, and (4) eradication. Besides these measures, control of environmental factors such as temperature and ventilation and use of solid partitions between pens is essential. Desinfection should be included in any control program; the organism is sensitive to a wide range of commonly used disinfectants (Gutierrez et al., 1995).

Antimicrobial treatment

Antimicrobial therapy is efficient only in the initial phase of the disease, when it can reduce mortality. For treatment of acutely affected animals antimicrobials should be applied in high dosage and parenterally (subcutaneously or intramuscularly), as affected animals have reduced food and water consumption. To ensure effective and durable blood concentrations, repeated injections may be required. A combination of parenteral and peroral medication in a recent outbreak often gives best results. The success of therapy depends mainly on early detection of clinical signs and on rapid therapeutic intervention. In some outbreaks, reappearance of the disease occurs a few weeks after cessation of peroral therapy. In these cases, pulse medication is sometimes recommended i.e. medication periods (during 4 or 5 days) interrupted by periods during which animals are not treated (during 4 or 5 days). The idea behind pulse medication is that during the non-treatment period animals build up immunity.

Both lung damage and fibrous pleurisy may persist affecting the performance for the remainder of the finishing period. Lesions may be colonised by organisms such as *Pasteurella* spp. and these may persist after *A. pleuropneumoniae* has been eliminated.

A. pleuropneumoniae is particularly susceptible *in vitro* to penicillin, amoxicillin, ampicillin, cephalosporin, tetracyclines, colistin, sulfonamide, combination of trimethoprim and sulfamethoxazole, and gentamicin. Higher minimum inhibitory concentrations (MIC) values are found for streptomycin, kanamycin, spectinomycin, spiramycin and lincomycin (Nicolet and Schifferli, 1982; Gilbride and Rosendal, 1984; Nadeau et al., 1988; Inoue et al., 1984; Homme et al., 1988). Gilbride and Rosendal (1984) and Vaillancourt et al. (1988) described that acquired resistance to ampicillin, streptomycin, sulfonamides and tetracyclines is frequent in serotypes 1, 3, 5 and 7 but rare in other serotypes, particularly serotype 2 (Nicolet and

Schifferli, 1982; Inoue et al., 1984). Acquired resistance to oxytetracycline has also been described by Dom et al. (1994a) in Belgian *A. pleuropneumoniae* strains belonging to serotypes 2, 3, 5b, 8 and 9. Antibiotic resistance is generally speaking plasmid mediated (Hirsh et al., 1982; Huether et al., 1987; Wilson et al., 1989).

The betalactams (penicillins and cephalosporins), trimethoprim and sulfamethoxazole, and tetracyclines are considered to be most active. Quinolone derivatives (enrofloxacin) (Kobisch et al., 1990) or the semi-synthetic cephalosporin ceftiofur sodium (Stephano et al., 1990) have been shown to be particularly effective after experimental challenge. Tiamulin and a combination of lincomycin and spectinomycin also gave satisfactory results in the field. Tilmicosin has been used in feed by Moore et al. (1996). Antimicrobial sensitivity testing is recommended where problems are being experienced with treatment.

An important disadvantage of antimicrobial use is that it favours spread of resistance not only in pathogenic bacteria but also in bacteria belonging to the normal flora. The latter can act as a reservoir for resistance genes. Moreover, penetration of antimicrobials in necrotic lesions often is not sufficient to eliminate the bacterium, resulting in persistence of *A. pleuropneumoniae* in these lesions.

Vaccination

Bacterins

Bacterins are inactivated whole-cell vaccines. They consist of bacterial cell suspensions adjusted to an opacity of some 10^{10} organisms per ml, usually inactivated by treatment with formaldehyde, UV-light, ozone or heat.

Vaccination with killed bacteria is serotype specific (Nielsen, 1984; Hensel et al., 1995a; Hensel et al., 1995b; Tarasiuk et al., 1994), but cross-immunity is possible with cross-reacting serotypes (Nielsen, 1985). Furthermore, Jolie et al. (1995) demonstrated that protection induced by bacterins is not only serotype specific but even can be subtype specific. The protection afforded can be extended by including all the serotypes present in a given geographic area.

Bacterins prepared with a 6 hour culture gave significant better results than bacterins prepared with a 24 hour culture (Nielsen, 1976). Data presented by Tarasiuk et al. (1994) indicate that there are no differences in efficacy between intramuscular and subcutaneous routes of vaccine administration.

A bacterin contains capsular polysaccharides and lipopolysaccharides (Furesz et al., 1997). Formaldehyde inactivation may alter certain antigens by denaturation. As a result, antibodies against these altered antigens may not recognize the original antigens. This could explain the limited, serotype specific protection of many bacterins in the field (Fedorka-Cray et al., 1990; Higgins et al., 1985; Nielsen, 1976).

The type of adjuvant used may affect efficacy. Bacterins containing an oil-adjuvant are superior in stimulating antibody production and protection. However, these vaccines are

frequently associated with tissue necrosis and abscess formation at the site of injection (Henry, 1983; Edelman, 1980; Straw et al., 1984).

A single oral administration of inactivated *A. pleuropneumoniae* provided partial clinical protection against a homologous challenge infection (Hensel et al., 1995b). Pigs exposed to vaccine aerosols of inactivated *A. pleuropneumoniae* biotype 1-serotype 2 or 9 had no clinical symptoms of pleuropneumonia or lung lesions when challenged with a serotype 9 strain (Hensel et al., 1996).

Subunit vaccines

Subunit vaccines are currently under development or being marketed and consist of varying combinations of subunits. A wide range of antigens has been found to be protective.

Immunisation of mice with a lipopolysaccharide containing vaccine resulted in partial protection against a homologous challenge with *A. pleuropneumoniae*. To increase the immune response to the polysaccharides, high molecular mass O-polysaccharides were chemically coupled to the large immunogenic protein bovine serum albumine (Rioux et al., 1997).

Bak et al. (1990) investigated the immunogenicity and the toxic effect of a vaccine containing purified capsular polysaccharides and a trace of lipopolysaccharides. Pigs immunized with this vaccine developed low antibody titers against these antigens. Instillation of large amounts of capsular material in pigs resulted in pulmonary oedema, hemorrhage and degenerative changes of alveolar epithelial cells.

It was demonstrated by Chiang et al. (1990) that vaccination of pigs with proteinase K-treated outer membrane fraction from *A. pleuropneumoniae* conferred better protection compared to vaccination with an untreated outer membrane fraction. Although pigs vaccinated with the proteinase K-treated outer membrane fraction vaccine had higher antibody levels against the outer membrane proteins no direct correlation was found between specific antibody levels and protection.

Neutralisation of the Apx toxins produced by *A. pleuropneumoniae* is very important in providing protection against pleuropneumonia. A vaccine containing a mixture of ApxI and ApxII induced partial protection against an *A. pleuropneumoniae* serotype 1 strain (Haga et al., 1997). Devenisch et al. (1990) and Crujisen et al. (1995) found a correlation between the amount of Apx-neutralizing antibodies and protection.

A vaccine containing a mixture of antigens can be used to induce better protection. Madsen et al. (1995) compared the effects of vaccination with outer membrane proteins, ApxI toxin or a combination of outer membrane proteins and ApxI toxin. It was concluded that (1) outer membrane proteins or Apx I toxin used individually induce incomplete protection; (2) outer membrane proteins plus Apx I toxin protect lung tissue better than when either virulence factor is used alone; (3) using outer membrane proteins plus ApxI toxin reduces the damage to lung tissue. Immunization with subunit conjugate vaccines (i.e. capsular polysaccharides conjugated to the ApxI toxin or lipopolysaccharides conjugated to the ApxI toxin) resulted in

antibody responses against each component of each conjugate (Byrd and Kadis, 1992). Pigs immunized with a trivalent vaccine containing ApxI toxin, ApxII toxin and a 42 kDa outer membrane protein were partially protected against challenge with different *A. pleuropneumoniae* serotypes (Van den Bosch et al., 1994).

Recombinant expressed ApxII and transferrin binding protein A of an *A. pleuropneumoniae* serotype 7 strain induced better protection against challenge with an *A. pleuropneumoniae* serotype 7 strain than when bacterins were used (Rossi-Campos et al., 1992).

Vaccination with a cell-free concentrate containing carbohydrates, endotoxin and protein with hemolytic and cytotoxic activity provided protection from mortality and significantly reduced morbidity to homologous challenge (Fedorka-Cray et al., 1993).

Live vaccines

Several studies were performed with live attenuated vaccines against *A. pleuropneumoniae*. Naturally occurring mutants or strains attenuated in the laboratory were used.

Strain BES is an naturally occurring low virulence strain of *A. pleuropneumoniae* serotype 1. No reversion to virulence has been observed after successive passages in live pigs. Intranasal vaccination of pigs with this strain resulted in an overall decrease of mortality and lung lesions (Utrera et al., 1990).

Inzana et al. (1993) treated *A. pleuropneumoniae* serotypes 1 and 5 with ethyl methanesulfonic acid. This resulted in live, attenuated, stable strains without any detectable capsule. These vaccines appeared to provide protection similar to that induced by the parent strain against homologous and heterologous serotype challenge.

Byrd and Hooke (1997) evaluated temperature-sensitive mutants of *A. pleuropneumoniae* in mice. Intranasal immunization with these mutants resulted in protection against challenge with the homologous wild-type strain.

Isolation-treatment-vaccination combination

Isolation using all-in, all-out systems within a farm reduces infection but does not prevent it. The use of vaccination or treatment prior to or at the move increases the degree of control (Taylor, 1999).

Eradication

Hysterectomy-derived herds are free of *A. pleuropneumoniae* infection and may be maintained free by isolation. They form a source of disease-free stock for repopulation.

Depopulation of farms, cleaning, disinfection and repopulation with disease-free animals can be carried out (Taylor, 1999). Farms free from the disease and infection should maintain a policy of isolation coupled with the use of semen or embryos to introduce new genes. Any animals introduced should be hysterectomy derived or originate from herds known to be free from the disease and from infection. It may be appropriate to hold them in quarantine prior to introduction to the herds (Taylor, 1999).

Antimicrobial treatment alone has not been successful in eradication but combinations with vaccination, partial depopulation and removal of serological and tonsillar carriers has been successful (Taylor, 1999).

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SCIENTIFIC AIMS

A. pleuropneumoniae causes severe losses in the pig rearing industry. Although the disease can be controlled by antimicrobial agents, the use of these products has several disadvantages including induction of acquired resistance in pathogenic bacteria and bacteria belonging to the normal flora of pigs. Therefore, prevention should be encouraged in the control of the disease. Vaccines could be very useful to control porcine pleuropneumonia. Rational design of effective antibacterial vaccines requires knowledge of the virulence factors of the bacterium and the pathogenesis of the disease.

It has been shown that Apx toxins play an important role in the pathogenesis of porcine pleuropneumonia. The first aim of this thesis was, therefore, to evaluate the efficacy of vaccines mainly based on the inclusion of these toxins.

Inclusion of bacterial adhesins in subunit vaccines might be of value. Indeed, *A. pleuropneumoniae* first adheres to alveolar epithelial cells before it causes lung lesions. The hypothesis was that, once the bacteria have attached to their target cells, i.e. alveolar epithelial cells, the Apx toxins are released directly onto the host cell. Hereby, neutralizing antibodies have no opportunity to bind to the toxins and prevent their action. Therefore, in the second part of this thesis, the purpose was to characterize the adhesion of *A. pleuropneumoniae* to alveolar epithelial cells *in vitro* and *in vivo*.

In a final study it was determined whether pigs vaccinated with a bacterin consisting of bacteria grown under conditions resulting in high *in vitro* adhesion, were better protected against an *A. pleuropneumoniae* infection than pigs vaccinated with a bacterin consisting of bacteria grown under conditions resulting in low *in vitro* adhesion.

EXPERIMENTAL STUDIES

CHAPTER 1. EVALUATION OF THE EFFICACY OF COMMERCIALLY AVAILABLE VACCINES AGAINST PLEUROPNEUMONIA

Effects of endobronchial challenge with *Actinobacillus pleuropneumoniae* serotype 9 of pigs vaccinated with inactivated vaccines containing the Apx toxins

Effect of endobronchial challenge with *Actinobacillus pleuropneumoniae* serotype 9 of pigs vaccinated with a vaccine containing Apx toxins and transferrin-binding proteins

CHAPTER 2. ADHESION OF *ACTINOBACILLUS PLEUROPNEUMONIAE* TO PORCINE ALVEOLAR EPITHELIAL CELLS *IN VITRO* AND *IN VIVO*

Characterization of the *in vitro* adhesion of *Actinobacillus pleuropneumoniae* to alveolar epithelial cells

Effect of culture conditions of *Actinobacillus pleuropneumoniae* serotype 2 and 9 strains on *in vivo* adhesion to alveoli of pigs

CHAPTER 3. EVALUATION OF THE EFFICACY OF A VACCINE CONTAINING CANDIDATE-ADHESINS

Effect of endobronchial challenge with *Actinobacillus pleuropneumoniae* serotype 10 of pigs vaccinated with bacterins consisting of *Actinobacillus pleuropneumoniae* serotype 10 grown under NAD-rich and NAD-restricted conditions

CHAPTER 1

EVALUATION OF EFFICACY OF COMMERCIALY AVAILABLE VACCINES AGAINST PLEUROPNEUMONIA

Effects of endobronchial challenge with *Actinobacillus pleuropneumoniae* serotype 9 of pigs vaccinated with inactivated vaccines containing the Apx toxins

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SUMMARY

The efficacy of two inactivated vaccines containing the Apx toxins of *Actinobacillus pleuropneumoniae* (Hemopig™, Biokema, Lausanne, Switzerland and Porcilis™ App, Intervet, Boxmeer, The Netherlands) was determined. Ten pigs were vaccinated twice with Hemopig™ and eight pigs with Porcilis™ App. Ten control animals were injected twice with a saline solution. Three weeks after the second vaccination, all pigs were endobronchially inoculated with 10^5 colony-forming units (CFU) of an *A. pleuropneumoniae* serotype 9 strain. Increased respiratory rate and/or fever were observed in all vaccinated and control pigs after challenge. One pig of the Hemopig™ group and of the Porcilis™ App group died, whereas all pigs of the control group survived the challenge. Surviving pigs were killed at 7 days after challenge. The mean percentage of affected lung tissue was 34% in the control group, 16% in the Hemopig™ group, and 17% in the Porcilis™ App group. *A. pleuropneumoniae* was isolated from the lungs of all 10 control animals, from 7 of the 10 animals vaccinated with Hemopig™ and from 5 of the 8 animals vaccinated with Porcilis™ App. The mean bacterial titres of the caudal lung lobes were 1.4×10^6 CFU/g in the control group, 1.7×10^3 CFU/g in the Hemopig™ group, and 4.8×10^3 CFU/g in the Porcilis™ App group. In both vaccinated groups the mean number of days with dyspnoea, the mean number of days with fever, the mean percentage of affected lung tissue, and the mean bacterial titre in the caudal lung lobes were significantly lower than in the control group. Significant differences between the two vaccinated groups were not observed. It was concluded that both vaccines induced partial protection.

INTRODUCTION

A. pleuropneumoniae is the causative agent of porcine pleuropneumonia which induces great economic losses in the pig-rearing industry. It also causes severe animal suffering and hampers animal welfare. Infected pigs may develop acute haemorrhagic-necrotizing pneumonia and fibrinous pleuritis or chronic localized lung lesions and adhesive pleuritis (Nicolet, 1992). For control of porcine pleuropneumonia, improvement of housing conditions and climate is essential. To control outbreaks of this disease, vaccination may also be useful. Although whole-cell bacterins may reduce mortality after infection with the homologous serotype, they generally do not confer protection against challenge with heterologous serotypes (Fenwick and Henry, 1994). An explanation for the limited protection might be the absence of secreted and certain bacteria-associated virulence factors in the bacterins. More recently, vaccines containing the *A. pleuropneumoniae*-RTX-toxins (Apx toxins) have become commercially available. Among the different serotypes of *A. pleuropneumoniae*, three of these exotoxins have been described and each serotype produces either one or two of them (Dom et al., 1994; Frey et al., 1993; Frey et al., 1994; Kamp et al., 1991). Specific pathogen-free (SPF) pigs vaccinated twice with a vaccine containing the Apx toxins and a 42-kDa outer membrane protein developed no or less severe clinical symptoms and lung lesions than non-vaccinated controls after challenge with serotype 1, 2, and 9 strains (Kobisch and Van den Bosch, 1992; Van den Bosch et al., 1992). Field trials carried out in France (Pommier et al., 1996), the Netherlands (Valks et al., 1996), and Italy (Martelli et al., 1996) confirmed that vaccination with this vaccine can result in reduction of clinical symptoms and lung lesions of acute and chronic pleuropneumonia and improvement of production parameters (growth, feed conversion, medication).

In the present study, the efficacy of two inactivated vaccines containing the Apx toxins of *A. pleuropneumoniae* was evaluated, using a well-standardized challenge model which results in the acute form of porcine pleuropneumonia (Dom and Haesebrouck, 1994; Haesebrouck et al., 1996).

MATERIALS AND METHODS

Challenge strain

The *A. pleuropneumoniae* biotype 1-serotype 9 strain (reference nr 13261) was used (Smits et al., 1991). Bacteria were grown for 6 hours (log phase of growth) on Columbia agar (Columbia Agar Base, Lab M, Bury, Great Britain) supplemented with 3% horse serum, 0.03% NAD (Sigma Chemical Co, St Louis, Mo, USA), and 5% yeast extract at 37°C in a humid atmosphere with 5% CO₂. Bacteria were harvested in phosphate-buffered saline solution (PBSS, pH 7.3), centrifuged at 400 x g for 20 minutes, and suspended in RPMI 1640 supplemented with 10% non-essential amino acids, 10% glutamine, 10% fetal calf serum, and 1% sodium pyruvate. The suspension was checked for purity and the number of colony-

forming units (CFU) was determined by plating tenfold dilutions on Columbia agar supplemented with 3% horse serum, 0.03% NAD, and 5% yeast extract. Bacterial suspensions were stored overnight at 4°C. The next day, they were used in the experiments.

Pigs

In these studies 28 pigs were used. All animals were obtained by using a medicated segregated early weaning programme. They were weaned at 18 days of age and kept in isolation until used in the experiments.

Vaccines

Two commercial subunit vaccines were used, Hemopig™ (Biokema S.A., Lausanne, Switzerland) and Porcilis™ App (Intervet, Boxmeer, The Netherlands). The Hemopig™ vaccine contains the capsular antigens of an *A. pleuropneumoniae* serotype 2, 7, and 9 strain, their Apx toxins, and the Apx toxins of a serotype 1 strain. The Porcilis™ App vaccine contains Apx I, II, and III toxins and a 42-kDa outer membrane protein (Van den Bosch et al., 1992).

Experimental design

At the age of 19 and 22 weeks, 10 pigs were injected subcutaneously with 4 ml of the Hemopig™ vaccine, 8 pigs were injected intramuscularly with 2 ml of the Porcilis™ App vaccine, and 10 pigs were injected subcutaneously with 4 ml of a saline solution. Three weeks after the second vaccination, all pigs were experimentally infected. The pigs were anaesthetized with azaperone 2 mg/kg IM (Stresnil®, Janssen Pharmaceutica, Beerse, Belgium) and thiopental 10 mg/kg IV (Pentothal®, Abott, Louvain-La-Neuve, Belgium). They were inoculated endobronchially with 10⁵ CFU of the *A. pleuropneumoniae* serotype 9 strain in 5 ml inoculum (Dom et al., 1992; Haesebrouck et al., 1996). All pigs were examined clinically. Pigs that died were autopsied immediately; those that survived the challenge were killed 7 days after inoculation. At necropsy, the lungs were examined macroscopically and the percentage of affected lung tissue was determined. For this purpose, a diagram was used that divides the lung into 74 equal triangles (Hannan et al., 1982). The percentage of affected lung tissue was determined by summation of the triangles showing pneumonia and/or pleuritis divided by 74. Samples from lungs, tracheobronchial lymph nodes, tonsils, liver, kidney, and spleen were taken for bacteriological examination. Sera were collected before the first and the second vaccination and before the challenge.

Clinical examination

The pigs were examined for signs of pneumonia, characterized by increased respiratory rate (>40 inspirations/min), dyspnoea, sneezing, coughing, and the presence of bloody foam on mouth and/or nostrils. Rectal temperature was measured 1 day before inoculation, just before inoculation, and during the 7 days after inoculation. Other indications of infection were vomiting and a depressed appearance.

Bacteriological examination

Twenty per cent (w/v) suspensions of the right and the left caudal lung lobes were made in PBSS. The number of CFU was determined by plating tenfold dilutions of the suspensions on Columbia agar supplemented with 3% equine serum, 0.03% NAD, and 5% yeast extract. Samples were also grown on Columbia agar supplemented with 5% bovine blood with a *Staphylococcus intermedius* streak. Twenty per cent (w/v) suspensions of tonsils were made in PBSS and inoculated onto blood agar with *S. intermedius*. Suspected colonies were subcultured on the same medium. Samples from lung lesions, tracheobronchial lymph nodes, liver, kidney, and spleen were tested for the presence of *A. pleuropneumoniae* by making an incision in these tissues and taking a sample with an inoculation loop. These samples were also inoculated onto blood agar with *S. intermedius*.

Serology

All pig sera were tested for neutralizing antibodies against Apx I, Apx II, and Apx III, using a bioassay based on neutral red uptake by viable pulmonary alveolar macrophages, as described previously (Dom et al., 1994).

Statistical analysis

Statistical analysis was performed on the following variables: mortality, morbidity (i.e. percentage of animals with dyspnoea and/or fever), percentage of animals with dyspnoea, mean number of days during which dyspnoea was observed, percentage of animals with fever, mean number of days during which fever was observed, mean percentage of affected lung tissue, and logarithmic mean of bacterial titre in caudal lung lobes.

Fisher's Exact test was used to compare proportions between groups, and the means were compared using the non-parametric Wilcoxon rank sum test.

RESULTS

Clinical examination

Disease signs were not observed before challenge and at the time of challenge. In the control group (n = 10), dyspnoea was observed 1 day and 2 days after challenge in 7 and 5 animals, respectively. The mean number of days during which dyspnoea was observed was 1.2 (Table 1). In 3, 4, 4, 2, 6, and 2 pigs the respiratory rate was increased on days 1, 2, 3, 4, 5, and 6 after the challenge, respectively. Sneezing or coughing was observed in all pigs of this group. All animals were depressed the first 3 days after the challenge. They were seen resting on the sternum and it was difficult to force them to move. Seven animals were still depressed 5 days after challenge. Fever (> 40°C) was detected in 10, 6, 6, 4, 5, 2, and 1 pig on days 1, 2, 3, 4, 5, 6, and 7, respectively. The mean number of days during which fever was observed was 3.4 (Table 1).

In the Hemopig™ group (n = 10), 1 pig died 1 day after challenge. In this pig, dyspnoea and coughing had been observed. In 2 other pigs dyspnoea was observed 1 day after challenge. The mean number of days during which dyspnoea was observed was 0.22 (Table 1). In all surviving pigs, an increased respiratory rate was observed the first 2 days after inoculation. Coughing was observed in 7 pigs. Five animals were depressed the first 3 days after challenge. At 1, 2, 3, 4, and 5 days after challenge fever was detected in 5, 5, 4, 4, and 1 pig, respectively. In 3 animals, fever was never detected after challenge. The mean number of days when fever was detected was 1.7 days (Table 1).

In the Porcilis™ App group (n = 8), 1 pig died 2 days after challenge. In this pig, dyspnoea and fever were present. Dyspnoea was observed in 1 of the 7 surviving pigs 2 days after challenge. The mean number of days during which dyspnoea was observed was 0.14 days (Table 1). The first 2 days after challenge, an increased respiratory rate was observed in all the surviving pigs. In 5 pigs coughing was detected. Six pigs were depressed during the first 2 days after challenge. Four, 1, 1, and 1 pig developed fever at 1, 2, 3, and 5 days after challenge, respectively. In 3 pigs, fever was not detected. The mean number of days when fever was detected was 1.0 day (Table 1).

The mean rectal temperatures of the pigs in the control group, the Hemopig™ group, and the Porcilis™ App group are compared in Figure 1.

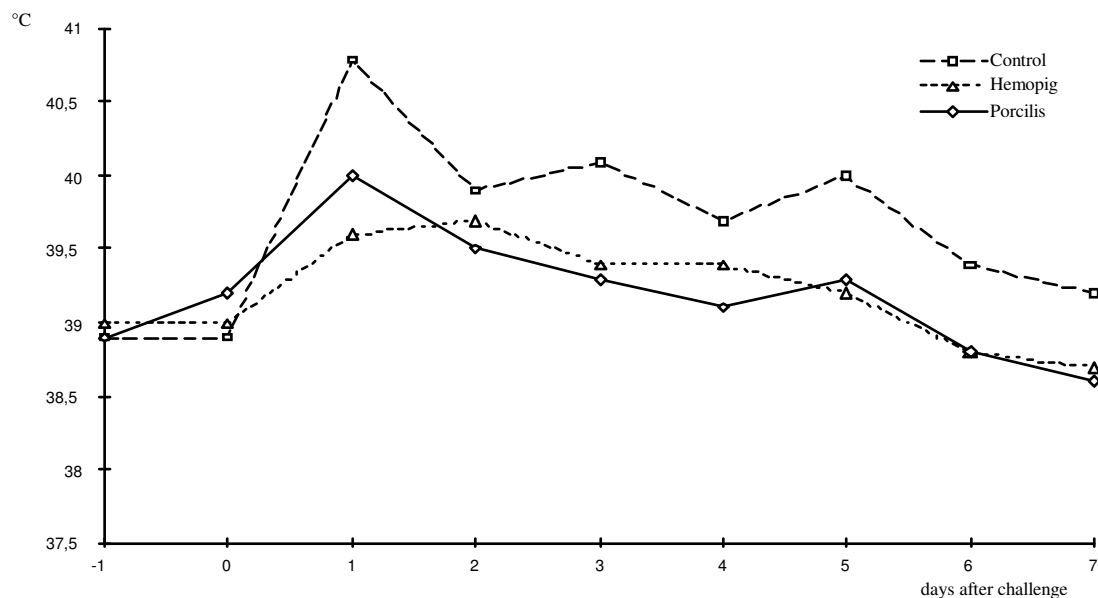


Figure 1: Mean body temperatures following challenge with *A. pleuropneumoniae* serotype 9 in control pigs (n=10), pigs vaccinated with Hemopig™ (n=10) or Porcilis™ App (n=8).

Necropsy findings

At necropsy, a haemorrhagic necrotizing pneumonia and fibrinous pleuritis were found in all piglets of the control group. The percentages of affected lung tissue varied from 20% to 73% (mean 34.4%; Table 1).

Lung lesions were not observed in 3 animals of the Hemopig™ group. In the pig that died, 58% of the lungs were affected. In the other animals that developed lung pathology, the percentage of affected lung tissue varied from 2 to 56% (mean: 16.1%; Table 1). The lung lesions were characterized by haemorrhagic necrotizing pneumonia.

In the Porcilis™ App group, lung lesions were not observed in 3 animals. In the pig that died, 51% of the lung tissue was affected. In the other animals with lung lesions, the percentage of affected lung tissue varied between 10% to 35% (mean: 16.9%; Table 1). Lesions were similar to those described above for the Hemopig™ group.

Bacteriology

The results are summarized in tables 1 and 2. In the control pigs, *A. pleuropneumoniae* was isolated from all lung lobes with lesions. The logarithmic mean bacterial titre of the caudal lobes was 1.4×10^6 CFU/g (Table 1). *A. pleuropneumoniae* was isolated from the tonsils of 3 pigs. The challenge strain could not be isolated from any of the other samples.

A. pleuropneumoniae was not isolated from the lungs of 3 animals vaccinated with Hemopig™. The logarithmic mean number of CFU/g lung tissue of the caudal lung lobes was 2.2×10^3 . The strain could also be isolated from the tonsils, tracheobronchial lymph nodes, and spleen of the pig that died. *A. pleuropneumoniae* was not isolated from the other samples.

A. pleuropneumoniae was not isolated from the lungs of 3 animals vaccinated with Porcilis™ App. The logarithmic mean bacterial titre of the caudal lobes was 4.8×10^3 CFU/g in this group. *A. pleuropneumoniae* was isolated from the tonsils of the pig that died. *A. pleuropneumoniae* was not isolated from other samples.

Serology

Antibodies against Apx I, Apx II, and Apx III were not detected in sera from pigs of the control group and in sera from vaccinated pigs collected at the time of first and second vaccination.

Antibody titres against Apx I and II at the time of challenge of the vaccinated pigs are presented in table 2. It can be seen that antibody titres against Apx I and II were either low or absent. In none of the animals were antibodies against Apx III detected.

Statistical analysis

Significant differences ($p < 0.05$) between the control group and both vaccinated groups were found for the mean number of days during which dyspnoea was observed, the mean number of days during which fever was detected, the mean percentage of affected lung tissue, and

the logarithmic mean bacterial titre in the caudal lung lobes (Table 1). Significant differences could not be demonstrated between the vaccinated groups.

Table 1: Results of endobronchial challenge with *A. pleuropneumoniae* serotype 9 in pigs vaccinated with Hemopig™ or Porcilis™ App and in non-vaccinated control pigs

	Control group (n = 10)	Hemopig™ group (n = 10)	Porcilis™ group (n = 8)
mortality (%)	0	10	13
morbidity ⁽¹⁾ (%)	100	100	100
animals with dyspnoea (%)	70	30	25
mean number of days with dyspnoea ⁽²⁾	1.20	0.22 *	0.14 *
animals with fever ⁽³⁾ (%)	100	60(4) *	63 *
mean number of days with fever	3.4	1.7 *	1.0 *
mean % of affected lung tissue	34.4	16.1 *	16.9 *
mean bacterial titre in caudal lung lobes ⁽⁵⁾ (CFU)	1.4x10 ⁶	1.7x10 ³ *	4.8x10 ³ *

(1). morbidity: % of animals with increased respiratory rate and/or fever

(2). only pigs that survived challenge were included

(3). fever: body temperature $\geq 40^{\circ}\text{C}$

(4). one animal died approximately 27 hours after infection. Temperature was only taken once. Fever was not observed.

(5). logarithmic mean: values of <100 CFU/g lung tissue were considered 101

* significant difference with control group (p <0.05)

Table 2: Results of pathology, serology, and bacteriology in pigs vaccinated with Hemopig™ or Porcilis™ App and in non-vaccinated animals after challenge with *A. pleuropneumoniae* serotype 9

Group	pig nr	antibody titre at the time of challenge against		Isolation of <i>A. pleuropneumoniae</i> from lungs	Bacterial titre in caudal lung lobes (CFU/gram)	% of affected lung tissue
		Apx I	Apx II			
Control	1	<20	<20	yes	4.0x10 ³	20
	2	<20	<20	yes	4.5x10 ⁷	47
	3	<20	<20	yes	1.3x10 ⁶	30
	4	<20	<20	yes	4.7x10 ⁶	28
	5	<20	<20	yes	2.0x10 ³	30
	6	<20	<20	yes	2.0x10 ⁵	20
	7	<20	<20	yes	1.0x10 ⁷	38
	8	<20	<20	yes	2.2x10 ⁸	27
	9	<20	<20	yes	6.5x10 ⁷	32
	10	<20	<20	yes	3.4x10 ⁵	73
Hemopig	11	<20	<20	yes	<10 ²	7
	12	<20	<20	yes	3.3x10 ⁵	13
	13	<20	<20	yes	8.0x10 ⁷	58
	14	20	<20	no	<10 ²	0
	15	<20	<20	yes	5.0x10 ⁴	12
	16	<20	<20	yes	4.0x10 ⁴	56
	17	80	40	no	<10 ²	0
	18	<20	<20	no	<10 ²	0
	19	<20	<20	yes	5.1x10 ⁴	12
	20	<20	<20	yes	<10 ²	2
Porcilis	21	20	<20	yes	1.7x10 ⁶	51
	22	20	<20	yes	4.6x10 ⁶	22
	23	20	<20	yes	1.2x10 ³	10
	24	20	<20	no	<10 ²	0
	25	20	<20	yes	1.5x10 ⁵	35
	26	<20	<20	no	<10 ²	0
	27	<20	<20	yes	2.0x10 ⁵	16.0
	28	40	<20	no	<10 ²	0

DISCUSSION

In the present study, an endobronchial challenge model was used. An advantage of this method is that it delivers an exact number of bacteria to the lungs, allowing standardization of experimental infections.

All control pigs developed acute disease and at necropsy, macroscopic pulmonary lesions typical of porcine pleuropneumonia were observed. None of these animals died, indicating that the challenge model was of planned severity.

The clinical signs of disease persisted for a shorter time in the vaccinated than in the control animals. Furthermore, lesions were more severe and the mean bacterial titre in lung tissue was higher in control animals, indicating partial protection in both vaccinated groups.

Although both vaccines contained Apx toxins, no or only low neutralizing antibody titres were detected in vaccinated animals. The reason for this finding is not clear. Interference with maternally derived antibodies can most probably be excluded, since the first vaccination was carried out at 19 weeks of age and antibodies against Apx toxins were not detected at the time of first vaccination. It is possible that the assay used here is of limited sensitivity, but in previous studies with the same assay, high toxin-neutralizing titres were detected in pigs infected with virulent *A. pleuropneumoniae* strains (Haesebrouck et al., 1996). In the latter studies, animals were completely protected against challenge with the homologous serotype. Further research is necessary to develop vaccines that induce high Apx toxin-neutralizing antibody titres.

It has been shown that the Apx toxins are essential vaccine components to confer protection against challenge with pathogenic *A. pleuropneumoniae* (Beaudet et al., 1994; Byrd et al., 1992; Van den Bosch et al., 1992). Although both vaccines used in the present study contained Apx toxins, they provided only partial protection against challenge carried out 3 weeks after the second vaccination. These results and the results of previous studies indicate that other bacterial components may also play a role in protection (Haesebrouck et al., 1996) and/or that higher titres are required for protection. It is possible that antibodies against the Apx toxins decrease the severity of the disease and allow animals to recover faster but do not prevent the initial infection. This might require antibodies against other bacterial compounds.

In the present studies, the protection obtained with the Porcilis™ App vaccine was lower than the protection described by Kobisch and Van den Bosch (1992) and Van den Bosch et al. (1992). In the latter studies, SPF pigs were used whereas in the study described here, conventional pigs were used. It is possible that protection induced in conventional pigs is more variable than that induced in SPF pigs.

In only 5 of the 28 animals was the challenge strain isolated from tonsils. The isolation of *A. pleuropneumoniae* from tonsils, however, is difficult. The media used for the isolation are often overgrown by the abundant microflora present in the tonsils. This complicates visual identification.

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Effect of endobronchial challenge with *Actinobacillus pleuropneumoniae* serotype 9 of pigs vaccinated with a vaccine containing Apx toxins and transferrin-binding proteins

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SUMMARY

The efficacy of a subunit vaccine containing the Apx toxins of *Actinobacillus pleuropneumoniae* and transferrin binding proteins was determined. Ten pigs were vaccinated twice with the vaccine. Eight control animals were injected twice with a saline solution. Three weeks after the second vaccination, all pigs were endobronchially inoculated with $10^{6.5}$ colony-forming units (CFU) of an *A. pleuropneumoniae* serotype 9 strain. In the vaccine group, none of the pigs died after inoculation. Only one pig of the control group survived challenge. Surviving pigs were killed at 7 days after challenge. The mean percentage of affected lung tissue was 64% in the control group and 17% in the vaccine group. *A. pleuropneumoniae* was isolated from the lungs of all animals. The mean bacterial titres of the caudal lung lobes were 5.0×10^8 CFU/g in the control group and 3.0×10^6 CFU/g in the vaccine group. It was concluded that the vaccine induced partial protection against severe challenge.

INTRODUCTION

A. pleuropneumoniae causes porcine contagious pleuropneumonia, which is distributed world wide and results in serious losses in the pig rearing industry. It also causes severe animal suffering. The disease is characterized, in the acute stage, by a hemorrhagic necrotizing pneumonia and fibrinous pleuritis. In the chronic stage, localized lung lesions and adhesive pleuritis can be seen (Nicolet, 1992). For control of porcine pleuropneumonia, improving housing conditions and climate is essential. To control outbreaks of this disease, vaccination may be useful. Although the so-called “first generation vaccines” of whole-cell bacterins may reduce mortality after infection with the homologous serotype, they generally do not confer protection against challenge with heterologous serotypes (Fenwick and Henry, 1994). An explanation for the limited protection might be the absence of secreted and certain bacteria-associated virulence factors in the bacterins. So-called “second generation vaccines” i.e. vaccines containing the *A. pleuropneumoniae* Apx toxins have become commercially available. Among the different serotypes of *A. pleuropneumoniae*, three of these exotoxins have been described and each serotype produces either one or two of them (Dom et al., 1994; Frey et al., 1993; Frey et al., 1994; Kamp et al., 1991). In a recent study, we demonstrated partial protection against endobronchial challenge in animals vaccinated twice with two second generation vaccines (Chiers et al., 1998). Field trials carried out in France (Pommier et al., 1996), the Netherlands (Valks et al., 1996), Italy (Martelli et al., 1996), Spain (López et al., 1998) and Norway (Lium et al., 1998) confirmed that vaccination with these vaccines can result in reduction of clinical symptoms and lung lesions of acute and chronic pleuropneumonia and improvement of performance (growth, feed conversion, cost of medication).

Apx toxins are not the only antigens involved in protection against porcine pleuropneumonia. Capsular antigens, cell wall lipopolysaccharides, outer membrane proteins, fimbriae and transferrin binding proteins also play a role (Haesebrouck et al., 1997).

In the present study, the efficacy of a vaccine, containing several recombinant cell-associated and secreted antigens of *A. pleuropneumoniae* including the Apx toxins and transferrin binding proteins, was determined using a severe, well standardized challenge model (Dom and Haesebrouck, 1992; Haesebrouck et al., 1996).

MATERIALS AND METHODS

Challenge strain

An *A. pleuropneumoniae* biotype 1-serotype 9 strain (N° 13261) was used (Smits et al., 1991). Bacteria were grown for 6 hours (log phase of growth) on Columbia agar (Columbia Agar Base, Lab M, Bury, Great Britain) supplemented with 3% horse serum, 0.03% NAD (Sigma Chemical Co, St Louis, Mo, USA), and 5% yeast extract at 37°C in a humid atmosphere with 5% CO₂. Bacteria were harvested in phosphate-buffered saline solution

(PBSS, pH 7.3), centrifuged at 400 x g for 20 minutes, and suspended in RPMI 1640 supplemented with 10% non-essential amino acids, 10% glutamine, 10% foetal calf serum, and 1% sodium pyruvate. The suspension was checked for purity and the number of colony-forming units (CFU) was determined by plating tenfold dilutions on Columbia agar supplemented with 3% horse serum, 0.03% NAD, and 5% yeast extract. Bacterial suspensions were stored overnight at 4°C. The next day, they were used in the experiments.

Pigs

In this study 18 pigs were used. All animals were obtained by using a medicated segregated early weaning programme (Chiers et al., 1998). They were weaned at 18 days of age and kept in isolation until used in the experiments.

Vaccine

An acellular pentavalent subunit vaccine prepared with recombinant antigens from *A. pleuropneumoniae* was used (Pleurostar™, Biostar Inc., Saskatoon, Canada). It contains transferrin binding proteins and Apx toxins.

Experimental design

At the age of 7 and 11 weeks, pigs were intramuscularly injected: 10 pigs with 2 ml of the vaccine and 8 pigs with 2 ml of a saline solution. Three weeks after the second vaccination, all pigs were experimentally infected. The pigs were anaesthetized with Zoletil® 100 (2.2 mg/kg tiletamine and 2.2 mg/kg zolazepam, Virbac, Louvain La Neuve, Belgium) and Xyl-M® 2% (4.4 mg/kg xylazine, VMD, Arendonk, Belgium). They were inoculated endobronchially with $10^{6.5}$ CFU of the challenge strain in 5 ml inoculum (Dom and Haesebrouck, 1992; Haesebrouck et al., 1996). All pigs were examined clinically. Pigs that died were autopsied immediately; those that survived the challenge were killed 7 days after inoculation. At necropsy, the lungs were examined macroscopically and the percentage of affected lung tissue was determined. For this purpose, a diagram was used that divides the lung into 74 equal triangles (Hannan et al., 1982). The percentage of affected lung tissue was determined by summing up the triangles showing pneumonia and/or pleuritis divided by 74. Samples from lungs, tracheobronchial lymph nodes, tonsils, liver, kidney, and spleen were taken for bacteriological examination. Sera were collected before the first and the second vaccination and before the challenge.

Clinical examination

The pigs were examined for signs of pneumonia, characterized by increased respiratory rate (>40 inspirations/min), dyspnoea, sneezing, coughing, and the presence of bloody foam on mouth and/or nostrils. Rectal temperature was measured 1 day before challenge inoculation, just before inoculation, and during the 7 days after inoculation. Other indications of infection were vomiting and a depressed appearance.

Bacteriological examination

Twenty per cent (w/v) suspensions of the right and the left caudal lung lobes were made in PBSS. The number of CFU was determined by plating tenfold dilutions of the suspensions on Columbia agar supplemented with 3% equine serum, 0.03% NAD and 5% yeast extract. Samples were also grown on Columbia agar supplemented with 5% bovine blood with a *Staphylococcus intermedius* streak. Twenty per cent (w/v) suspensions of tonsils were made in PBSS and inoculated onto blood agar with *S. intermedius*. Suspected colonies were subcultured on the same medium. Samples from lung lesions, tracheobronchial lymph nodes, liver, kidney and spleen were tested for the presence of *A. pleuropneumoniae* by making an incision in these tissues and taking a sample with an inoculation loop. These samples were also inoculated onto blood agar with *S. intermedius*.

Serology

All pig sera were tested for presence of neutralising antibodies against Apx I, II and III using a bioassay based on neutral red uptake by viable pulmonary alveolar macrophages, as described previously (Dom et al., 1994).

Statistical analysis

Statistical analysis was performed on the following variables: mortality, morbidity (i.e. percentage of animals with dyspnoea and/or fever), percentage of animals with fever, percentage of animals with dyspnoea, mean percentage of affected lung tissue and logarithmic mean of bacterial titre in caudal lung lobes. Fisher's exact test was used to compare proportions between groups. The mean percentages of affected lung tissue were compared using the non-parametric Kruskal-Wallis test and the mean bacterial titres in caudal lung lobes were compared using an ANOVA test.

RESULTS

Results are summarized in Table 1.

Clinical examination

None of the animals showed disease signs before challenge and at the time of challenge. In the control group (n=8), six pigs were found dead 20 h after inoculation. One pig died 48h after inoculation. In this pig, fever, dyspnoea, coughing and depression were present. The remaining pig presented fever (>40°C), increased respiratory rate and coughing during 6 days after challenge and was depressed on day 2.

In the vaccinated group (n=10), none of the pigs died after inoculation. In 1, 1, 4 and 1 pig, fever was detected during 1, 2, 5 and 6 days after challenge, respectively. The mean number of days during which fever was observed was 3.1. In 4, 1 and 1 pigs, increased respiratory rate was observed during 1, 2 and 3 days, respectively. None of the animals showed

dyspnoea. In four pigs occasional coughing was observed. One animal with increased respiratory rate was depressed.

Necropsy findings

In all animals lung lesions, when present, were characterized by a haemorrhagic necrotizing pneumonia and fibrinous pleuritis .

In 7 animals of the control group more than 45% of the lung tissue and in one animal 31% of the lung tissue was affected (variation 31-88%; mean: 64%).

In eight animals of the vaccinated group the % of affected lung tissue was lower than 20%. In two animals more than 45% of the lung tissue was affected. The percentages of affected lung tissue varied from 4% to 53% (mean 17%).

Bacteriology

The results are summarized in Table 1.

In the control pigs, *A. pleuropneumoniae* was isolated from all lung lobes with lesions. The number of CFU/gram lung tissue of the caudal lobes varied between 4.0×10^6 and 4.5×10^9 , with a logarithmic mean of 5.0×10^8 . The challenge strain was isolated from the tonsils and tracheobronchial lymphnodes of 8 and 5 pigs, respectively. *A. pleuropneumoniae* was not isolated from liver, spleen or kidney of any animals in this group.

A. pleuropneumoniae was isolated from all lung lobes with lesions and from all tonsils of the vaccinated animals. The number of CFU/gram lung tissue of the caudal lobes varied between 2.7×10^4 and 1.1×10^8 , with a logarithmic mean of 3.0×10^6 . *A. pleuropneumoniae* was not isolated from tracheobronchial lymphnodes, liver, spleen or kidney of any animals in this group.

Serology

At the time of first vaccination, antibody titres against Apx I, II and III varied between <20 and 80. At the time of second vaccination, most animals were negative for antibodies against the Apx toxins. Low titres (20) were detected in 2 and 3 animals of the control and vaccinated group, respectively. Antibody titres against Apx I, II and III at the time of challenge varied between <20 and 40.

Statistical analysis

The vaccinated group differed significantly ($p < 0.05$) from the control group for mortality, percentage of animals with dyspnoea, percentage of affected lung tissue and logarithmic mean of bacterial titre in the caudal lung lobes.

Table 1. Results of endobronchial challenge with *A. pleuropneumoniae* serotype 9 in vaccinated pigs and in non-vaccinated control pigs

	Control group (n=8)	vaccine group (n=10)
morbidity ⁽¹⁾ (%)	100	70
mortality (%)	88	0*
% animals with dyspnoea ⁽²⁾	100	0*
% animals with fever ^(2, 3)	100	70
mean % of affected lung tissue	64	17*
mean bacterial titre in caudal lung lobes (CFU/g)	5.0×10^8	$3.0 \times 10^{6*}$

(1) morbidity: % of animals with increased respiratory rate and/or fever

(2) only pigs that survived more than 24 hours after challenge were included

(3) fever: body temperature $\geq 40^\circ\text{C}$

* significant difference with control group ($p < 0.05$)

Table 2. Results of pathology, serology and bacteriology in vaccinated pigs and in non-vaccinated control animals after challenge with *A. pleuropneumoniae* serotype 9

Group	pig nr	antibody titre at the time of challenge against			Bacterial titre in caudal lung lobes (CFU/gram)	% of affected lung tissue
		Apx I	Apx II	Apx III		
Control	1	<20	<20	<20	4.0×10^6	75
	2	<20	<20	<20	1.0×10^9	57
	3	<20	<20	<20	1.9×10^9	88
	4	<20	<20	<20	4.5×10^9	71
	5	<20	<20	<20	2.2×10^9	54
	6	<20	<20	<20	6.8×10^7	76
	7	<20	<20	<20	5.4×10^8	60
	8	<20	<20	<20	1.3×10^9	31
Vaccinated	18	<20	20	<20	3.1×10^7	7
	19	<20	20	<20	5.2×10^5	4
	20	20	20	<20	1.1×10^8	17
	21	<20	40	<20	2.0×10^6	5
	22	20	20	<20	2.7×10^4	5
	23	40	<20	<20	1.5×10^7	53
	24	40	<20	<20	3.4×10^5	5
	25	40	<20	<20	2.5×10^7	45
	26	40	<20	<20	3.0×10^5	11
	27	40	<20	<20	3.5×10^7	18

DISCUSSION

Intranasal inoculation or aerosol exposure closely resemble the natural infection route of *A. pleuropneumoniae*. These methods are difficult to standardize, however, because coughing, sneezing, swallowing, breathing and mucociliary clearance can reduce the number of bacteria that reach the alveolar region. In the present study, an endobronchial challenge model was used. An advantage of this model is that it delivers an exact number of bacteria into the bronchi, allowing standardization of experimental infections. Furthermore, this method allows us to bring the bacteria directly close to the multiplication site of *A. pleuropneumoniae* namely the alveoli and bronchioli (Haesebrouck et al., 1997). A possible disadvantage is that the challenge strain is applicated not all over the lung but given more focal. Endobronchial challenge is therefore more likely to break through low level immunity in the respiratory tract than a more natural challenge route.

Seven of the 8 control animals died within 48 hours after infection. This demonstrates that a severe challenge model was used.

None of the vaccinated animals died and none of them developed dyspnoea. Furthermore, the mean % of affected lung tissue and the mean bacterial titre in the caudal lung lobes were significantly lower than in the control animals. This indicates that this vaccine induced partial protection against severe challenge.

It has been shown that the Apx toxins are essential vaccine components to confer protection against challenge with *A. pleuropneumoniae* (Beaudet et al., 1994; Byrd et al., 1992; Van den Bosch et al., 1992). However, in a previous study using a milder challenge model resulting in no mortality in non-vaccinated control pigs, animals vaccinated with vaccines containing Apx toxins were only partially protected against *A. pleuropneumoniae* serotype 9. It was suggested that antibodies against other bacterial components are also required for protection (Chiers et al., 1998). The vaccine used in the present studies also contained transferrin binding proteins and induced significant protection against a very severe challenge. This finding is in agreement with a study performed by Rossi et al. (1992). They found that pigs vaccinated with a recombinant protein subunit vaccine containing Apx II toxin and a transferrin binding protein of an *A. pleuropneumoniae* serotype 7 strain showed only minimal clinical signs or lung lesions after a homologous challenge.

Although the vaccine contained Apx toxins, only low neutralizing antibody titres were detected in the vaccinated animals. The reason for this finding is not clear. Interference with maternally derived antibodies can not be excluded, since the first vaccination was carried out at 7 weeks of age and low antibody titres against Apx toxins were detected at the time of first vaccination. Furthermore, it is possible that the assay used here was of limited sensitivity, although in previous studies with the same assay, high toxin-neutralizing titres were detected in pigs infected with virulent *A. pleuropneumoniae* strains (Haesebrouck et al., 1996). In the latter studies, animals were completely protected against challenge with the homologous serotype. Further research is necessary to develop vaccines that induce high Apx toxin-neutralizing antibody titres.

In the present studies, animals were only challenged with a serotype 9 strain. Since Apx toxins and transferrin binding proteins were included in the vaccine, protection against other serotypes can be expected (Van den Bosch et al., 1992; Wilke et al., 1997). This should, however, be confirmed in challenge studies with other serotypes.

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CHAPTER 2

ADHESION OF *ACTINOBACILLUS* *PLEUROPNEUMONIAE* TO PORCINE ALVEOLAR EPITHELIAL CELLS *IN VITRO* AND *IN VIVO*

Characterization of the *in vitro* adhesion of *Actinobacillus pleuropneumoniae* to swine alveolar epithelial cells

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SUMMARY

A. pleuropneumoniae biovar 1 serotype 2, 5a, 9 and 10 strains were tested for their ability to adhere to alveolar epithelial cells in culture. For the serotype 5a, 9 and 10 strains, optimal adherence was observed after growth of bacterial cells in a NAD-restricted medium (0.001% NAD). This condition was also associated with the expression of a 55 kDa outer membrane protein and of fimbriae. For the serotype 2 strain, adherence and expression of fimbriae and a 55 kDa outer membrane protein was less influenced by the growth conditions. The N-terminal amino acid sequence of the 55 kDa outer membrane protein had no homology with any known sequence, suggesting that it is an as yet unknown protein. Adherence capabilities were significantly reduced following treatment of the bacteria with proteolytic enzymes or heat. These findings suggest that proteins are involved in adhesion. The hydrophobic bond breaking agent tetramethylurea was unable to inhibit the adherence of *A. pleuropneumoniae* to alveolar epithelial cells. Treatment of the bacteria with sodium metaperiodate resulted in lower adhesion scores for the serotype 2 and 9 strains but the inhibition of adhesion was clearly lower than after treatment with proteolytic enzymes. This indicates that, besides proteins, carbohydrates might also be involved in adhesion of *A. pleuropneumoniae* to alveolar epithelial cells. The finding that inhibition of adhesion was very high when bacteria were treated with a combination of sodium metaperiodate and pronase also suggests that more than one adhesin is involved.

INTRODUCTION

Actinobacillus pleuropneumoniae is a Gram-negative bacterium causing contagious pleuropneumonia in pigs. Based on NAD requirements, *A. pleuropneumoniae* can be divided into biovar 1 strains, which are NAD-dependent, and biovar 2 strains, which are NAD-independent. So far 15 serotypes have been described (Blackall et al., 2002). Serotypes 1 and 5 are subdivided into 1a, 1b and 5a, 5b (Jolie et al., 1994; Nielsen, 1986).

In previous studies, it was shown that *A. pleuropneumoniae* adheres to the epithelium of the alveoli or the cilia of the terminal bronchioli of experimentally infected pigs (Dom et al., 1994). It has also been shown that *A. pleuropneumoniae* adheres to porcine tracheal rings maintained in culture (Bélanger et al., 1990), to porcine frozen tracheal and lung sections (Jacques et al., 1991), to erythrocytes from various animal species (Jacques et al., 1988), to tonsillar epithelial cells (Chiers et al., 1999), to swine buccal epithelial cells (Hamer et al., 1999) and to type III swine-lung collagen (Enriquez et al., 1999).

Adherence to host tissue is generally regarded as an important prerequisite for colonization and virulence manifestation of bacteria (Ofek and Beachy, 1980). Adherence enables colonization to occur and allows the bacterium to exert its pathogenic effects. Several surface structures have been described in the family of *Pasteurellaceae* that are involved in adhesion, including the capsule (Confer et al., 1995), fimbriae (Read et al., 1992), lipopolysaccharides (Bélanger et al., 1990) and outer membrane proteins (Confer et al., 1995). Concerning *A. pleuropneumoniae*, only few adhesion factors have been described. Lipopolysaccharides seem to be involved in the *in vitro* adhesion to porcine tracheal rings (Bélanger et al., 1990) and mucus (Bélanger et al., 1992; 1994). Recently, it was shown that the capsule is not responsible for adherence to tracheal frozen sections (Jacques, 1999). Type 4 fimbriae have been shown on *A. pleuropneumoniae* (Dom et al., 1994a; Zhang et al., 2000) but their role in adherence needs to be elucidated.

Since *in vivo* studies indicated that adherence of *A. pleuropneumoniae* to alveolar epithelial cells constitutes an important initial step in pathogenesis (Dom et al., 1994; Haesebrouck et al., 1997), the association of the bacterium with these host cells was here further characterized, using an *in vitro* adhesion model.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Reference strains of *A. pleuropneumoniae* serotype 9 (13261) and 10 (D13039), as well as a serotype 2 (266/7656) and 5a (1736) strain, both obtained from the lungs of pigs that died during outbreaks of pleuropneumonia in Belgium (Dom et al., 1994b), were used in this study. All 4 strains were NAD-dependent (biovar 1). Stock suspensions were stored at -70°C in RPMI 1640 medium supplemented with 10% non-essential amino acids (Gibco Europe, Merelbeke, Belgium), 10% glutamine, 10% foetal calf serum (FCS) and 1% sodium pyruvate (Gibco) (leukocyte medium). To study the influence of culture conditions on adhesion,

bacteria were grown on Columbia agar supplemented with 1% horse serum and 0.001% NAD for 20 hours at 37°C (NAD-restricted medium), on Columbia agar supplemented with 3% horse serum, 0.001% NAD and 5% yeast extract for 20 hours at 37°C (NAD-restricted medium with yeast extract) or on Columbia agar supplemented with 3% horse serum, 0.03% NAD and 5% yeast-extract for 6 hours at 37°C (NAD-rich medium). To study the expression of surface antigens, bacteria were grown on the NAD-restricted and NAD-rich media described above. In all other studies, bacteria were grown for 20 hours on the NAD-restricted medium. All incubations were done in an atmosphere with 5% CO₂. The number of colony forming units (CFU) was determined by turbidimetry (optical density at 450 nm).

Adherence assay

Alveolar epithelial cells (AEC) were obtained as previously described (Van de Kerkhof et al., 1996). For the adherence assay, 10⁵ viable cells in 100 µl of supplemented Modified Eagle Medium (MEM, Gibco) were added to wells of 24-well microtiter plates with coverslips and incubated for 3 hours at 37°C in an atmosphere with 5% CO₂. Then, 1 ml of supplemented MEM was added to each well and the plates were further incubated for 2 days. Subsequently, plates were washed once with phosphate buffered saline solution (PBSS) and cells were fixed with 1 ml of methanol 100%. After washing the cells with distilled water, adhesion tests were performed. In these tests, 10⁸ CFU of the *A. pleuropneumoniae* strains in 1 ml inoculum were added to the cultures. The microtiter plates were centrifuged at 110×g for 10 minutes at 37°C and further incubated at 37°C for 30 minutes in an atmosphere with 5% CO₂. The plates were washed 4 times with PBSS, stained with Haemacolor[®] staining reagents (Merck, Darmstadt, Germany) and microscopically examined.

In each assay, one hundred cells were examined for the presence of adhering bacteria. The following scores were used for each alveolar epithelial cell: score 0 = no adhesion, score 1 = 1 to 20 bacteria adhered to the cell, score 2 = more than 20 bacteria adhered to the cell. The number of alveolar epithelial cells with score 0, 1 or 2 was determined.

Influence of culture conditions on adhesion to AEC and expression of capsule, fimbriae and outer membrane proteins

Influence of culture conditions on adhesion

To study the influence of the culture conditions on adhesion, adhesion tests were performed with *A. pleuropneumoniae* serotype 2, 5a, 9 and 10 strains grown on either the NAD-restricted medium, NAD-restricted medium with yeast extract or NAD-rich medium. These experiments were performed at least 3 times.

Influence of culture conditions on expression of capsule

A. pleuropneumoniae serotype 2, 5a and 9 strains were grown on the NAD-restricted and the NAD-rich medium. After incubation, bacteria were harvested, washed once with PBSS and resuspended in PBSS at a concentration of 10⁸ CFU/ml. In order to avoid collapse of the

capsules during the electron microscopic examination, bacterial suspensions were exposed to undiluted homologous antiserum for 1 hour at 4°C. The antisera were raised in rabbits against whole bacterial cells. The titers of agglutinating antibodies of the rabbit antisera varied between 1:8 and 1:32 (Russel W.M.S. and Burch R.L., 1959). Bacterial cells were then suspended in cacodylate buffer (0.1M, pH 7.0) containing 5% glutaraldehyde and 0.15% (w/v) ruthenium red. Fixation was done for 2 hours at 20°C. Thereafter, bacterial cells were immobilized in 3% agar, washed five times in cacodylate buffer with 0.05% ruthenium red and postfixed with 1% osmium tetroxide and 0.05% ruthenium red for 2 hours. Washings were repeated as above, and the samples were dehydrated in a graded series of acetone washes. All the solutions used in processing the specimen, from the wash after glutaraldehyde fixation to dehydration with the 70% acetone solution, contained 0.05% ruthenium red. Samples were then washed twice in propylene oxide and embedded in Spurr low-viscosity resin. Thin sections were cut and stained with uranyl acetate and lead citrate. They were examined with a transmission electron microscope (Philips 201) at an accelerating voltage of 60 kV. The capsule thickness was measured on micrographs taken during the electron microscopic observations. The cell wall thickness was measured between the inner edge of the capsule layer and the outer leaflet of the plasma membrane.

Influence of culture conditions on expression of fimbriae

A. pleuropneumoniae serotype 2, 5a, 9 and 10 strains were grown on the NAD-restricted and the NAD-rich medium, washed once in PBSS and then suspended in a minimal amount of distilled water at a concentration of 10^8 CFU/ml. The cultures were examined for the presence of fimbriae by negative staining and shadowing. For negative staining, a Formvar-coated copper grid was floated on the surface of the bacterial suspension for 10 minutes. The grid was then floated for 10 seconds on a solution of 2% uranyl acetate in distilled water. After staining, the grid was washed twice and examined with a Philips 201 transmission electron microscope (TEM). For shadowing, the bacterial suspension was absorbed on carbon coated grids and rotary shadowed with platinum-carbon at a small angle (5-6°) with the Balgers shadowing apparatus. The grid was examined with a Philips 201 TEM. The size of the fimbriae was measured on the micrographs taken during electron microscopic observations.

Influence of culture conditions on expression of outer membrane proteins

Sarcosyl insoluble outer membrane proteins (OMPs) of *A. pleuropneumoniae* serotype 2, 5a, 9 and 10 strains grown on the NAD-restricted and the NAD-rich medium were prepared (Deneer and Potter, 1989). Briefly, bacteria were suspended in 100 ml of 10 mM HEPES buffer (pH 7.5, Gibco, Ghent, Belgium) at a concentration of 2×10^9 CFU/ml. Cells were disrupted by ultrasound with 10 bursts, 20 seconds each, with cooling on ice. Debris was removed by centrifugation at $2,000 \times g$ for 20 minutes and 50 ml 2% (w/v) sarcosyl (Sigma Chemical Co, St Louis, Mo, USA) was added to the supernatant. After incubation for 10 minutes at room temperature, the outer membrane fraction was pelleted by centrifugation at

60,000×g for 1 hour and 40 minutes. The pellet was suspended in 10 ml 10 mM HEPES buffer and treated with 10 ml 2% sarcosyl for 20 minutes at room temperature. The OMP fraction was again pelleted by centrifugation at 60,000×g for 1 hour and 40 minutes and suspended in 1 ml of 10mM HEPES. Before storage at -70°C, the protein concentration was determined (BIORAD Protein Assay, BIORAD, Brussels, Belgium). After thawing, proteins (5 µg) of each sample were separated using discontinuous sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE).

Electrophoresis experiments were performed with a 4.5% stacking gel and a 12% running gel. Each antigen preparation was mixed with equal volumes of solubilization buffer [4% (w/v) of SDS, 10% (v/v) 2-mercapto ethanol, 17.4% (v/v) glycerol and 0.01% bromophenol blue in 0.06M Tris HCl (pH 6.8)] and boiled for 5 minutes prior to electrophoresis. After electrophoresis, silver staining was performed (Morrissey J., 1981).

N-terminal amino acid sequence analysis of an outer membrane protein

The OMPs of the *A. pleuropneumoniae* serotype 5a and 10 strains grown on the NAD-restricted medium were separated by SDS-PAGE, blotted onto a polyvinylidene difluoride membrane and stained with Coomassie blue. A 55 kDa OMP band was cut out and put in a blotcartridge (cross-flow type). The N-terminal amino acid sequence was determined in an automatic pulsed liquid phase sequencer (model 476, PE Biosystems, Foster City, Ca) using the Edman degradation method. Thereafter, a BLAST (Basis Local Alignment Search Tool) search was performed in the following databases: non-redundant GenBank CDS translations, PDB, SwissProt, Spupdate and PIR.

Effect of pretreatment of bacterial cells with enzymes, heat, sodium metaperiodate, tetramethylurea and carbohydrates on adhesion to alveolar epithelial cells

A. pleuropneumoniae serotype 2, 5a and 9 strains were grown on the NAD-restricted medium and suspended in PBSS at a concentration of 10⁸ CFU/ml. The suspensions were subjected to the following treatments: (1) bacteria were incubated for 30 minutes at 37°C with pepsin solution [1 mg/ml pepsin in 50 mM citrate-10 mM phosphate buffer (pH 3)]; (2) bacteria were incubated for 1 hour at 37°C with trypsin solution [1 mg/ml trypsin in PBSS (pH 7.5)]. Thereafter, the activity of trypsin was stopped by adding soybean trypsin inhibitor (200 U/ml; Sigma) in a sodium phosphate buffer (pH 7.6); (3) bacteria were incubated for 1 hour at 37°C in PBSS (pH 7.3) followed by an incubation for 40 minutes at 37°C in pronase solution [0.01M sodium acetate and 0.005M calcium acetate buffer (pH 7.5) containing 1 mg/ml pronase]; (4) bacteria were incubated for 1 hour at 37°C in a sodium metaperiodate solution [10 mg/ml sodium metaperiodate in PBSS (pH 7.3)] followed by an incubation for 40 minutes at 37°C in the sodium acetate and calcium acetate buffer without pronase; (5) bacteria were incubated for 1 hour at 37°C in the sodium metaperiodate solution followed by an incubation for 40 minutes at 37°C in the pronase solution; (6) bacteria were heated at 56°C for 10, 20 or 30 minutes; (7) bacteria were incubated for 1 hour at 37°C with the following carbohydrates: D-

arabinose, D-maltose, D-saccharose, D-mannose, D-glucosamine, D-galactose, N-acetyl-D-galactosamine, D-xylose or D-glucose (all carbohydrates at a final concentration of 0.2M); (8) bacteria were incubated for 1 h at 37°C with 0.01M of the hydrophobic bond-breaking agent 1, 1, 3, 3-tetramethylurea (TMU; Sigma Chemical Co., St. Louis, MO, USA.).

Controls consisted of non-heat treated bacteria or bacteria incubated in the buffers without pepsin, trypsin, pronase, sodium metaperiodate, carbohydrates or tetramethylurea. After the treatments with pepsin, trypsin, pronase, sodium metaperiodate or carbohydrates, bacterial suspensions were washed in PBSS, resuspended with PBSS to 10^8 bacteria/ml and used in the adhesion tests. After the treatment with tetramethylurea, adhesion tests were performed in the presence of tetramethylurea.

For each treatment, after incubation, the influence on the viability of the bacterial cells was determined by plating tenfold dilutions on Columbia agar supplemented with 3% horse serum, 0.03% NAD and 5% yeast extract and comparing the number of CFU/ml with controls.

All parts of the experiment were performed at least 2 times.

Statistical analysis

The influence of the culture conditions and the different pretreatments of the bacterial cells on adhesion were compared with the Kruskal-Wallis one way non-parametric analysis of variance test (computer program STATISTIX 4.1, Analytical software). A significance level of 0.05 was used.

RESULTS

Influence of culture conditions on adhesion and expression of capsule, fimbriae and outer membrane proteins

Influence of culture conditions on adhesion

The number of alveolar epithelial cells with adhesion scores 0, 1 or 2 is given in Table 1 for *A. pleuropneumoniae* serotype 2, 5a, 9 and 10 strains grown on the NAD-rich medium, the NAD-restricted medium and the NAD-restricted medium with yeast extract. For the serotype 5a, 9 and 10 strains, adhesion scores were significantly higher when bacteria were grown on both NAD-restricted media. For the serotype 2 strain, there was no significant difference in adhesion scores between the growth conditions.

Bacteria did not cover the whole surface of the alveolar epithelial cells; they only surrounded the alveolar epithelial cells (Figure 1).

Table 1. Influence of culture conditions on adhesion of *A. pleuropneumoniae* serotype 2, 5a, 9 and 10 strains to porcine alveolar epithelial cells.

Serotype	Growth conditions	Total number of alveolar epithelial cells	Number (%) of alveolar epithelial cells with score:		
			0 0 bacteria adhering	1 1-20 bacteria adhering	2 >20 bacteria adhering
2	NAD-restricted	700	0 (0)	83 (12)	617 (88)
	NAD-restricted with yeast extract	300	0 (0)	4 (1)	296 (99)
	NAD-rich	1800	0 (0)	268 (15)	1532 (85)
5a	NAD-restricted ^c	600	0 (0)	212 (35)	388 (65)
	NAD-restricted with yeast extract ^c	300	0 (0)	2 (1)	298 (99)
	NAD-rich ^{a, b}	500	239 (48)	261 (52)	0 (0)
9	NAD-restricted ^c	1200	42 (4)	400 (33)	758 (63)
	NAD-restricted with yeast extract ^c	300	0 (0)	29 (10)	271 (90)
	NAD-rich ^{a, b}	700	700 (100)	0 (0)	0 (0)
10	NAD-restricted ^c	100	141 (13)	770 (70)	189 (17)
	NAD-restricted with yeast extract ^c	300	57 (19)	198 (66)	45 (15)
	NAD-rich ^{a, b}	600	401 (67)	199 (33)	0 (0)

a = significant different from NAD-restricted group

b = significant different from NAD-restricted with yeast extract group

c = significant different from NAD-rich group

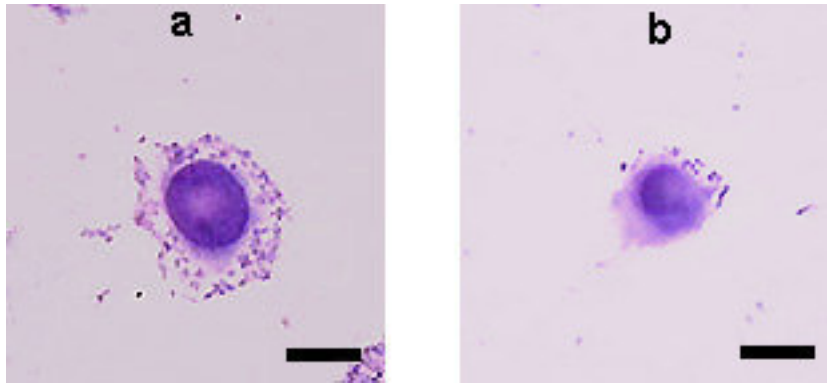


Figure 1: Light micrograph of alveolar epithelial cells incubated with an *A. pleuropneumoniae* serotype 9 strain grown on NAD-restricted (a) and NAD-rich (b) media. A high degree of adhesion is observed when bacteria were grown on NAD-restricted media: alveolar epithelial cells are surrounded by these bacteria. Bar = 10 µm.

Influence of culture conditions on expression of capsule

Bacterial cells of *A. pleuropneumoniae* serotype 2, 5a and 9 strains were covered with a layer of capsular material (Figure 2a and 2b). The thickest capsular layer (40-100 nm) was seen around cells from the serotype 2 and 5a strains grown in NAD-rich conditions (Figure 2a). These capsules had a regular, continuous appearance. The capsule of these strains was thinner and more irregular when grown in NAD-restricted conditions (20 to 50 nm) (Figure 2b). The serotype 9 strain had an irregular capsule with a thickness between 20 to 80 nm irrespective of the growth conditions.

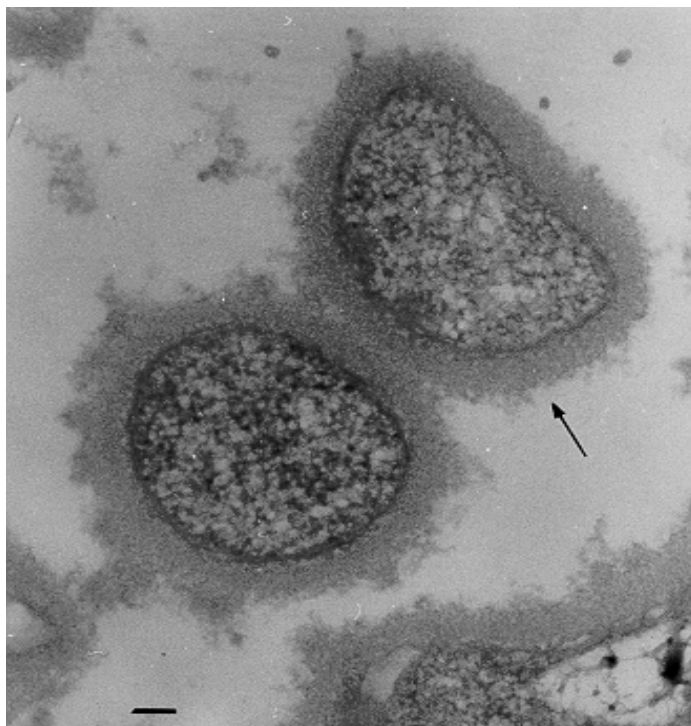


Figure 2a: Transmission electron micrograph of thin sections of *A. pleuropneumoniae* serotype 5a cells grown in NAD-rich conditions. Cells were stabilized with whole-cell homologous antiserum. Arrow shows capsular material. Bar = 200 nm.

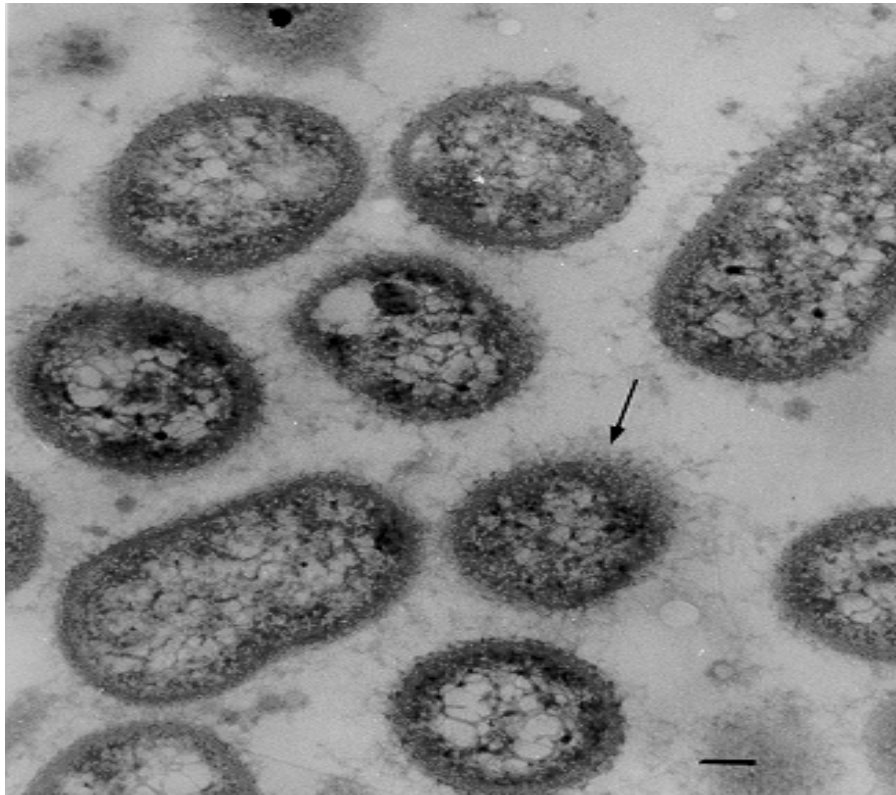


Figure 2b: Transmission electron micrograph of thin sections of *A. pleuropneumoniae* serotype 5a cells grown in NAD-restricted conditions. Cells were stabilized with whole-cell homologous antiserum. Arrow shows capsular material. Bar = 200 nm.

Influence of culture conditions on expression of fimbriae

Fimbriae extending outward from the cell walls were demonstrated on all *A. pleuropneumoniae* strains grown on the NAD-restricted medium and on the *A. pleuropneumoniae* serotype 2 strain grown on the NAD-rich medium. The fimbriae were most easily observed when rotary shadowing was used. They were filamentous, had a length of 400-800 nm and a width of 5-8 nm (Figure 3). The density of the fimbriae varied from cell to cell but was in general low. Not all cells in a culture were fimbriated. Fimbriae as described above could not be detected on the serotype 5a, 9 and 10 strains grown on the NAD-rich medium.

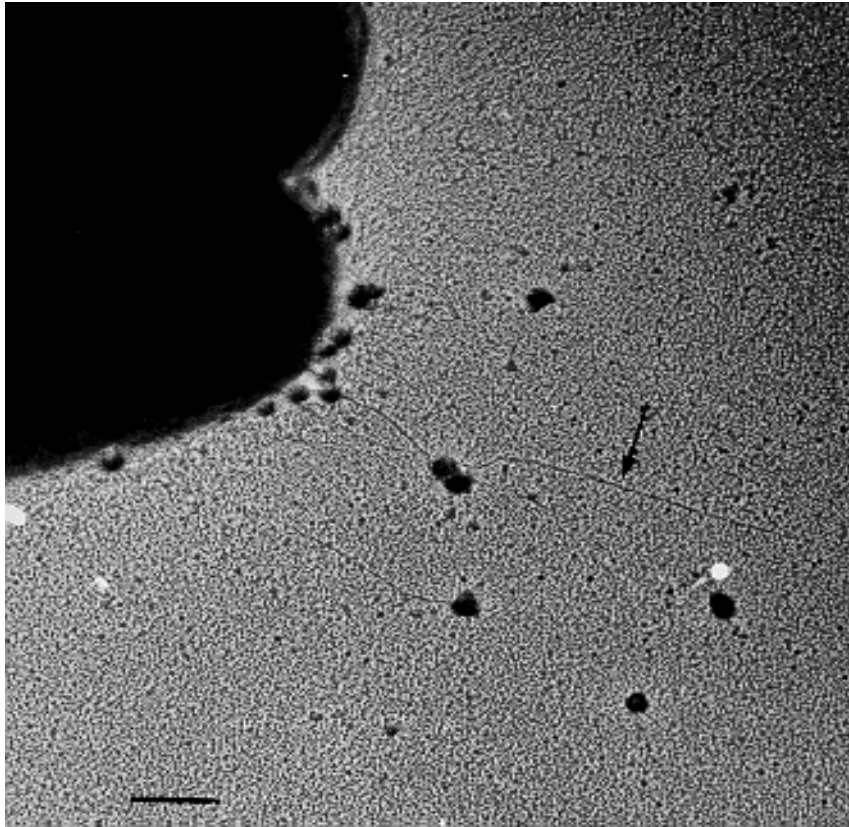


Figure 3: Transmission electron micrograph of the *A. pleuropneumoniae* serotype 2 strain grown on NAD-restricted medium. Long and thin filamentous appendages are indicated with arrow. Bar = 200 nm.

Influence of culture conditions on expression of outer membrane proteins

SDS-PAGE analysis of the sarcosyl-insoluble OMP fraction of *A. pleuropneumoniae* serotype 2, 5a, 9 and 10 strains grown on the NAD-rich or on the NAD-restricted medium is shown in Figure 4. In the OMP fraction of the serotype 5a, 9 and 10 strains, a 55 kDa band was observed when bacteria were grown in NAD-restricted conditions. This band was absent or much weaker when bacteria were grown in NAD-rich conditions. The 55 kDa band was present in the OMP fraction of the serotype 2 strain under both growth conditions.

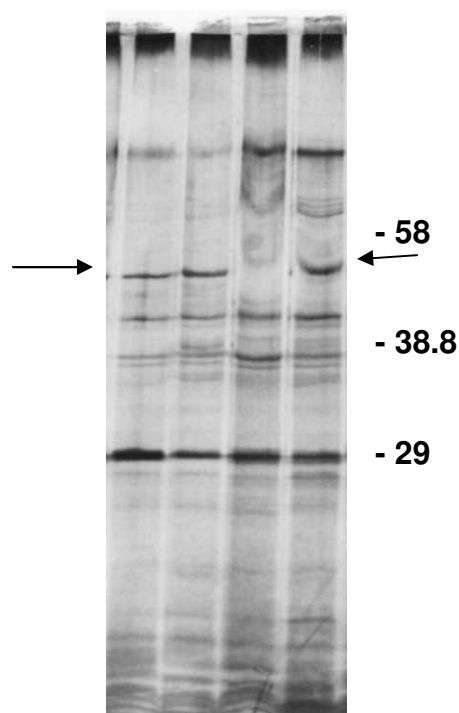


Figure 4: Silver-stained SDS-PAGE profiles of outer membrane protein fractions of *A. pleuropneumoniae* strains. Lanes: 1, serotype 2 strain grown on NAD-rich medium; 2, serotype 2 strain grown on NAD-restricted medium; 3, serotype 5a strain grown on NAD-rich medium; 4, serotype 5a strain grown on NAD-restricted medium. Arrows indicate 55 kDa band. The molecular masses are indicated on the right.

N-terminal amino acid sequence analysis

For the analysis of the N-terminal amino acid sequence of the 55 kDa OMP of the *A. pleuropneumoniae* serotype 5a and 10 strains, twenty-six cycles were run. For both serotypes, the following main sequence was found: Gly-Lys-Asp-Leu-Asn-Val-Phe-Asp-Lys-Asn-Tyr-Gly-Leu-Leu-Ile-Asn-Gly-Lys-(Gln)-Thr-Gln/Ala-Phe-Arg-Ser-Gly/Asp-Asp. No homology with other protein sequences was found in the databases.

Effect of pretreatment of bacterial cells with enzymes, heat, sodium metaperiodate, tetramethylurea and carbohydrates on adhesion to alveolar epithelial cells

Results are presented in Table 2. Treatment of the bacterial cells with enzymes, heat and sodium metaperiodate significantly decreased the adhesion of the serotype 2 strain. Treatments could be arranged in descending degree of inhibition as follows: combination of sodium metaperiodate and pronase, heat, pronase, pepsin, trypsin and sodium metaperiodate. For the serotype 5a strain, single sodium metaperiodate treatment had no significant effect on the adhesion. Treatment of the bacterial cells with enzymes and heat significantly decreased the adhesion. The highest inhibition was found with heat and a

combination of sodium metaperiodate and pronase followed by pronase, pepsin and trypsin. For the serotype 9 strain, all treatments significantly inhibited the adherence. In descending order, inhibition was observed with heat, combination of sodium metaperiodate and pronase, pronase, pepsin, trypsin and sodium metaperiodate.

Tetramethylurea or carbohydrate treatment did not reduce the level of adhesion. Pronase, trypsin, TMU and carbohydrate treatment of the bacterial cells did not have any effect on the viability. Treatment of the bacteria with heat, pepsin and sodium metaperiodate resulted in a decrease in viability.

Table 2. Effect of pretreatment of the bacterial cells with heat, enzymes and sodium metaperiodate on the adhesion of *A. pleuropneumoniae* strains to alveolar epithelial cells

% of alveolar epithelial cells with score 0, 1 or 2 after treatment of bacteria with											
Serotype	Scores*	No treatment	Pepsin	Trypsin	Pronase	Heat 10 min	Heat 20 min	Heat 30 min	Sodium metaperiodate	Sodium metaperiodate + pronase	Pronase + sodium metaperiodate
2	0	0	11	5	20	16	8	6	1	76	9
	1	12	66	69	72	84	88	93	71	24	77
	2	88	23	25	8	0	5	1	28	0	14
			a	a	a, b, c, h	a, b, c, h, j	a, c, h	a, b, c, h	a	a, b, c, d, e, f, g, h, j	a, h
5a	0	18	38	22	61	54	39	76	0	72	12
	1	50	54	63	39	46	61	24	62	28	57
	2	32	8	15	0	0	0	0	38	0	31
			a, c	a	a, b, c, f	a, b, c	a, c	a, b, c, f		a, b, c, f	
9	0	1	24	7	48	76	68	49	0	54	17
	1	37	58	60	49	24	32	51	51	46	73
	2	62	18	3	3	0	0	0	49	0	10
			a, h, c	a, h	a, b, c, h, j	a, b, c, d, g, h, i, j	a, b, c, d, h, j	a, b, c, h, j	a	a, b, c, h, j	a, c, h

* 0: 0 bacteria adhering; 1: 1-20 bacteria adhering; 2: >20 bacteria adhering

a-j: significant difference with no treatment (a), pepsin (b), trypsin (c), pronase (d), heat 10 min (e), heat 20 min (f), heat 30 min (g), sodium metaperiodate (h), sodium metaperiodate + pronase (i), pronase + sodium metaperiodate (j)

DISCUSSION

In previous studies, it has been shown that *A. pleuropneumoniae* adheres to alveolar epithelial cells of experimentally infected pigs (Dom et al., 1994 & 1995). Most probably, adherence of *A. pleuropneumoniae* to lower respiratory tract epithelial cells constitutes an important initial step in pathogenesis. Therefore, in the present work, porcine alveolar epithelial cells were used to study the adhesion of *A. pleuropneumoniae*. The alveolar epithelial cells were allowed to attach to coverslips whereafter they were fixed with methanol. Fixation was used to prevent destruction of the alveolar epithelial cells by the Apx toxins of *A. pleuropneumoniae* (Van de Kerkhof et al., 1996).

Advantages of using an *in vitro* system include reproducibility between assays, ease and simplicity of experimentation and animal welfare. However, there are also several disadvantages. Surfaces of *in vitro* cells may present proteins different from *in vivo* cells, especially in terms of distribution and accessibility of the receptors. Notably, the surfactant layer covering the alveolar epithelial cells *in vivo* is absent in the *in vitro* adhesion model. For this reason, data obtained in *in vitro* studies should be confirmed by *in vivo* studies.

The functional expression of most bacterial adhesins is not an essential activity for the survival of the bacteria. In fact, most organisms display differences in adhesive qualities during growth. These qualities can be modulated by pH and composition and physical state of growth medium (Ofek and Doyle, 1994). In this study, three growth media were used: a medium containing 0.03% NAD which was defined as NAD-rich and two media containing 0.001% NAD which were defined as NAD-restricted. For the serotype 5a, 9 and 10 strains, we found that growth on the NAD-restricted media resulted in a higher degree of adhesion, indicating that adhesins are more expressed. NAD-restricted conditions may reflect the *in vivo* situation. It is generally accepted that *A. pleuropneumoniae* is inhaled and enters the lung alveoli directly via the trachea and bronchi. In the lumen of the lung alveoli, extracellular NAD-concentrations most probably are very low, since, based on molecular weight and charge, it is unlikely that leakage of NAD out of normal living cells occurs. The NAD-restricted conditions in the lumen of lung alveoli may stimulate the expression of adhesins. Adhesion of *A. pleuropneumoniae* to alveolar epithelial cells may lead to a local high concentration of Apx toxins at the surface of host cells resulting in lysis of these cells (Van de Kerkhof et al., 1996) and release of growth components in the environment which may favor multiplication of the bacteria in lung tissue. The adhesion degree of bacteria belonging to the serotypes 5a, 9 and 10 strains grown for 6 hours on the NAD-rich medium was very low, indicating that adhesins were less expressed or less exposed at the bacterial surface.

Since adhesins should be located at the surface of the bacteria, electron microscopic examination and sodium dodecyl sulphate-polyacrylamide gel electrophoresis of outer membrane proteins was performed to study the surface components of the bacteria. For the serotype 5a, 9 and 10 strains, production of fimbriae and a 55 kDa outer membrane protein were observed when bacteria were grown under NAD-restricted conditions. These conditions

also resulted in highest *in vitro* adhesion scores to alveolar epithelial cells. This relationship between adherence levels and specific cell surface components might indicate that these components could play a role in *A. pleuropneumoniae* adherence to alveolar epithelial cells.

In contrast to the *A. pleuropneumoniae* serotype 5a, 9 and 10 strains, a correlation between the growth conditions and the adhesion of the serotype 2 strain was not observed. This strain exhibited a high degree of *in vitro* adhesion grown under NAD-restricted and NAD-rich conditions. Furthermore, fimbriae and a 55 kDa OMP were expressed under both growth conditions. This confirms that these components could play a role in *in vitro* adhesion.

Recently, fimbriae of *A. pleuropneumoniae* were characterized as type 4 fimbriae (Zhang et al., 2000). Type 4 fimbriae have been described in several other Gram-negative bacteria including *Haemophilus influenzae*, *Moraxella bovis*, *Pasteurella multocida* and *Pseudomonas aeruginosa*. A large number of studies have shown that they mediate attachment of these bacteria to epithelial cells *in vivo* and *in vitro* (Sato and Okinaga, 1987; Doig et al., 1988; Paranchych and Frost, 1988; Starr et al., 1999). Attempts should be made to try to isolate these fimbriae from *A. pleuropneumoniae* and determine their role in adhesion.

The outer membrane is an excellent compartment for anchoring and positioning adhesins to the outer surface. It has been demonstrated that outer membrane proteins, unrelated to fimbriae, can play a role in the adhesion to animal cells. Examples of such interaction have been found in the adhesion reactions of *Neisseria gonorrhoeae* (Dekker et al., 1990) and *Pseudomonas aeruginosa* (Ramphal et al., 1991). Recently, a 80 kDa OMP has been described that can play a role in the adherence of *A. pleuropneumoniae* to type III swine lung collagen (Enriquez et al., 1999). In the absence of lesions, collagen is not present on the surface of the alveoli, therefore adherence to collagen most probably is not involved in the initial adhesion of *A. pleuropneumoniae* to alveolar epithelial cells *in vivo*.

Bacteria were grown for 20 hours on the NAD-restricted medium and only 6 hours on the NAD-rich medium. Since the *A. pleuropneumoniae* strains used in this study were NAD-dependent, more time was needed for their growth on the NAD-restricted medium. Differences in incubation time could result in phenotypic variations of the strains. However, in preliminary studies, no differences in adhesion scores were observed between bacteria grown for 6 or 20 hours on the NAD-rich medium (data not shown). Therefore, most likely, the differences in adhesion observed in the present study were due to the altered growth media.

For many Gram-negative bacteria it has been shown that deprivation of nutrients results in the alteration of the protein composition of their outer membrane (Brown and Williams, 1985). In the present study, *A. pleuropneumoniae* serotype 2, 5a, 9 and 10 strains were deprived of NAD, resulting in a decreased growth for all strains and the expression of a 55 kDa OMP in serotype 5a, 9 and 10 strains. O'Reilly et al. (1991) demonstrated the expression of OMPs of 17, 31 and 69 kDa in *A. pleuropneumoniae* under pyridine nucleotide restricted conditions. OMPs in the relative molecular size range of 55 kDa were also described in other studies. OMPs of 54 kDa (Niven et al., 1989), 56 kDa (Gonzalez et al., 1990) and 60 kDa (Archambault et al., 1999) were induced when cells were grown under iron-restricted

conditions. The 60 kDa OMP was identified as a transferrin binding protein (Gerlach et al., 1992). Since the media used in this study were not deprived of iron, most likely the 55 kDa OMP does not correspond to an iron-regulated OMP. Furthermore, our finding that no homology in N-terminal amino acid sequence is present in the databases suggests that the 55 kDa OMP we observed has not yet been described before.

The capsule of *A. pleuropneumoniae* is generally regarded as an important virulence factor. It protects the pathogen against phagocytosis and lysis by complement (Haesebrouck et al., 1997). Capsular polysaccharides are not responsible for adherence to tracheal frozen sections, since a higher degree of adhesion was observed with non-capsulated mutant strains (Bélanger et al., 1990; Jacques et al., 1991; Rioux et al., 2000). Furthermore, Bélanger et al. (1992, 1994) demonstrated that heavily encapsulated *A. pleuropneumoniae* strains showed no or less affinity for porcine respiratory tract mucus than strains with a thinner capsule. From these studies, it was concluded that the capsule masks surface components, such as LPS, responsible for the *in vitro* adhesion to tracheal frozen sections and mucus. In the present study, the *A. pleuropneumoniae* serotype 5a strain grown under NAD-restricted conditions had a more irregular, thinner capsule and a higher adhesion score. This could confirm the finding that a thinner capsule may expose several adhesins. However, the serotype 9 strain had a thin, irregular capsule irrespective of the growth conditions and a high degree of adhesion to alveolar epithelial cells was only observed when the strain was grown on the NAD-restricted medium. This finding indicates that growth conditions, besides affecting the adhesion by altering the production of capsular material, may also influence the expression of surface antigens that are directly involved in adhesion.

In the present study, treatment of bacteria with tetramethylurea had no effect on the binding. Furthermore, hydrophobicity tests as described by Vercauteren et al. (1993) did not show differences in hydrophobicity between bacteria grown under NAD-restricted and NAD-rich conditions (data not shown). This indicates that, although hydrophobic interactions may contribute in a subordinate way to the overall adherence mechanism, it is unlikely that hydrophobicity per se is a major factor in adherence of *A. pleuropneumoniae* to alveolar epithelial cells.

Using the *in vitro* adhesion model, it was noted that the adherence capacity was strongly inhibited by treating the bacteria with proteolytic enzymes or heat. These findings suggest that proteins are involved in the adhesion. Treatment of the bacteria with sodium metaperiodate resulted in lower adhesion scores for the serotype 2 and 9 strains but the inhibition of adhesion was clearly lower than after treatment with proteolytic enzymes. Sodium metaperiodate is known to cleave the C-C bond between neighbouring hydroxyl groups of sugar. This indicates that, besides proteins, carbohydrates could also be involved in the adhesion of *A. pleuropneumoniae* to alveolar epithelial cells. Our finding that inhibition of adhesion was very high when bacteria were treated with a combination of sodium metaperiodate and pronase also suggests that more than one adhesin is involved.

In conclusion, we present several lines of evidence that more than one adhesin is involved in the *in vitro* adhesion of *A. pleuropneumoniae* to alveolar epithelial cells and that proteins play a major role. The fimbriae and the 55 kDa OMP, both of protein nature, can be designated as candidate-adhesins since bacteria expressing these structures had the highest adhesion scores. The expression of these components was higher under NAD-restricted conditions for the *A. pleuropneumoniae* serotype 5a, 9 and 10 strains, but not for the serotype 2 strain.

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Effect of culture conditions of *Actinobacillus pleuropneumoniae* serotype 2 and 9 strains on *in vivo* adhesion to alveoli of pigs

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SUMMARY

The effect of culture conditions on adhesion of inactivated *A. pleuropneumoniae* biotype 1-serotype 2 and 9 strains to alveoli of experimentally inoculated piglets was studied. Growing the bacteria on a NAD-restricted or NAD-rich medium did not influence this adhesion and had no effect on the *in vitro* association of the bacteria with surfactant proteins. Adhesion scores in alveolar epithelial cell cultures were, however, much higher when the serotype 9 strain was grown on the NAD-restricted medium. It is possible that different adhesins are involved in colonisation of alveoli of infected pigs. Association with surfactant might be a first step that precedes adhesion to alveolar epithelial cells.

INTRODUCTION

Actinobacillus pleuropneumoniae (*A. pleuropneumoniae*) is a Gram-negative bacterium causing contagious pleuropneumonia in pigs. Based on NAD requirements, *A. pleuropneumoniae* can be divided into biotype 1 strains, which are NAD-dependent, and biotype 2 strains, which are NAD-independent. So far 15 serotypes have been described (Blackall et al., 2002).

In previous studies it was shown that adhesion of *A. pleuropneumoniae* to alveoli is an important first step in the pathogenesis of porcine pleuropneumonia (Dom et al., 1994a&b). The adhesion was further characterized in alveolar epithelial cell cultures (Van Overbeke et al., 2002). For most *A. pleuropneumoniae* strains tested, it was shown that bacterial growth conditions influence *in vitro* adhesion to alveolar epithelial cells. Growth of *A. pleuropneumoniae* biotype 1-serotype 5a, 9 and 10 strains under NAD-restricted conditions resulted in more bacteria adhering to alveolar epithelial cells, indicating that a higher percentage of the bacterial population expresses adhesins under these conditions. *In vitro* however, cells may present proteins on their surface different from *in vivo* cells, especially in terms of distribution and accessibility of receptors. Moreover, the surfactant layer covering the alveolar epithelial cells *in vivo* may mask certain receptors on the cell surface. It has been reported that lipopolysaccharides of several bacteria including *Salmonella* serotype Typhi, *Klebsiella pneumoniae*, *Serratia marcescens* and *Pseudomonas aeruginosa* interact with surfactant (Brogden et al., 1986).

It was the aim of the present studies to determine if bacterial growth conditions that influence *in vitro* adhesion to porcine alveolar epithelial cells, also influence adhesion of *A. pleuropneumoniae* to porcine alveolar epithelial cells *in vivo*.

MATERIALS AND METHODS

Strains and culture conditions

Two *A. pleuropneumoniae* strains belonging to serotype 2 (strain 266/7656) and 9 (strain CVI 13261) and one *Escherichia coli* strain (32KH85) were used in this study. Stock suspensions were stored at -70°C in RPMI 1640 medium supplemented with 10% non-essential amino acids (Gibco Europe, Merelbeke, Belgium), 10% glutamine, 10% foetal calf serum (FCS) and 1% sodium pyruvate (Gibco) (leukocyte medium). The *A. pleuropneumoniae* bacteria were grown on Columbia agar supplemented with 1% horse serum and 0.001% NAD for 20 hours at 37°C (NAD-restricted medium) or on Columbia agar supplemented with 3% horse serum, 0.03% NAD and 5% yeast-extract for 6 hours at 37°C (NAD-rich medium). Previous studies demonstrated that adhesion of the serotype 9 strain to alveolar epithelial cell cultures is much higher when bacteria are grown in the NAD-restricted medium. *In vitro* adhesion of the serotype 2 strain to alveolar epithelial cell cultures is not influenced by these culture

conditions (Van Overbeke et al., 2002). All incubations were done in an atmosphere with 5% CO₂. The *E. coli* strain was grown on Mc Conkey agar for 20 hours at 37°C. Then, bacteria were harvested in phosphate buffered saline solution (PBSS), washed once and resuspended in PBSS at a concentration of 10⁹ colony forming units/ml (cfu/ml). To prevent *in vivo* expression of adhesins, bacteria were inactivated with UV before they were used in the experiments. This suspension was used as inoculum. Inactivation was checked by plating the bacterial suspensions onto Columbia agar supplemented with 5% bovine blood.

In vivo adhesion of A. pleuropneumoniae to alveolar epithelial cells of pigs

Twenty-seven three-week old Caesarean derived and colostrum deprived pigs were used. They were kept in isolation to prevent cross infections with other bacteria or viruses.

At the age of 3 weeks, pigs were anaesthetized with 2.2 mg/kg tiletamine and 2.2 mg/kg zolazepam (Zoletil® 100, Virbac, Louvain La Neuve, Belgium) and 4.4 mg/kg xylazine (Xyl-M® 2%, VMD, Arendonk, Belgium). They were intubated and endobronchially inoculated using an endoscope with 1 ml inoculum in the right lung and 1 ml inoculum in the left lung: 8 pigs were inoculated with the *A. pleuropneumoniae* serotype 9 strain grown on the NAD-rich medium, 5 pigs with the *A. pleuropneumoniae* serotype 9 strain grown on the NAD-restricted medium, 6 pigs with the *A. pleuropneumoniae* serotype 2 strain grown on the NAD-rich medium, 6 pigs with the *A. pleuropneumoniae* serotype 2 strain grown on the NAD-restricted medium and 2 pigs with 10⁹ CFU of the *E. coli* strain. The pigs were kept asleep until they were euthanized at 30 minutes post-inoculation. The right and left lung were separated and 5 samples of the inoculation sites of each lung were taken for histological examination.

The samples were processed for paraffin sectioning according to standard procedures. Sections were stained with Giemsa. The percentage of bacterial adherence was determined by lightmicroscopical examination of the sections. For each lung, the number of adhering bacteria was counted on a total of at least 500 observed bacteria (Dom et al., 1994b).

Interaction of A. pleuropneumoniae with surfactant proteins

Pig-derived lung surfactant (Curosurf®, Serono Benelux, Den Haag, The Netherlands), consisting of approximately 99% polar lipids (mainly phospholipids) and 1% hydrophobic, low molecular weight proteins (surfactant-associated proteins B and C) was used. The surfactant-associated proteins were biotinylated with a protein biotinylation kit (Amersham international, Buckinghamshire, UK).

One ml of the inactivated *A. pleuropneumoniae* biotype 1-serotype 2 and 9 strains grown on the NAD-rich and the NAD-restricted medium was centrifuged at 400 x g for 10 minutes. The supernatant was discarded and different amounts (80 µg, 40 µg, 20 µg, 10 µg, 5 µg, 2.5 µg or 1.25 µg) of biotinylated surfactant proteins were added to the pellet of bacteria. The mixture was incubated at 37°C for 1 hour. Thereafter, bacteria were washed twice with PBSS and resuspended in 50 µl of a 1:50 dilution of streptavidine-fluorescein isothiocyanate

(streptavidine-FITC) in PBSS. After another hour of incubation at 37°C, bacteria were washed twice with PBSS. Finally, samples were examined with a fluorescence microscope (Leica DM RBE, Brussel, Belgium).

Controls consisted of *A. pleuropneumoniae* biotype 1-serotype 2 and 9 strains grown on the NAD-rich and the NAD-restricted medium incubated with streptavidine-FITC.

RESULTS

In vivo adhesion of A. pleuropneumoniae to lung alveolar epithelial cells

A. pleuropneumoniae serotype 2 and 9 strains grown on the NAD-restricted or NAD-rich media were able to adhere to alveolar cells. The mean percentage of bacteria adhering to the alveolar epithelial cells was 72% and 66% for the serotype 9 strain grown on the NAD-rich medium or the NAD-restricted medium, respectively. For the serotype 2 strain, 59% of the bacteria grown on the NAD-rich medium and 77% of the bacteria grown on the NAD-restricted medium were found in association with alveoli.

Adhesion was not observed in pigs inoculated with the *E. coli* strain.

Interaction of A. pleuropneumoniae with surfactant proteins

Fluorescence was not detected in controls consisting of the *A. pleuropneumoniae* biotype 1-serotype 2 and 9 strains incubated with streptavidine-FITC.

Incubation of the *A. pleuropneumoniae* biotype 1-serotype 2 and 9 strains with biotinylated surfactant and streptavidine-FITC resulted in a clear fluorescence, indicating that surfactant associated proteins bound to the bacteria. The surfactant proteins bound to the serotype 2 and 9 strains grown on the NAD-restricted or NAD-rich media. The fluorescence decreased when lower concentrations of biotinylated surfactant proteins were used.

DISCUSSION

Intranasal inoculation or aerosol exposure closely resembles the natural infection route of *A. pleuropneumoniae*. These methods are difficult to standardize, however, because coughing, sneezing, swallowing, breathing and mucociliary clearance can reduce the number of bacteria that reach the alveolar region. In the present study, the bacteria were inoculated endobronchially using an endoscope. Advantages of this approach are that it delivers an exact number of bacteria in the bronchi and it delivers the bacteria at a chosen place in the lung. This allows standardization of the experimental inoculations. Pigs were anaesthetized before the inoculation and were kept asleep until they were euthanized. Coughing, sneezing and swallowing was hereby avoided. It is to be expected that a certain amount of bacteria are eliminated by breathing. However, this reduction of the number of bacteria is probably equivalent for the different serotypes.

In the present studies, *A. pleuropneumoniae* serotype 2 and 9 strains grown on the NAD-restricted or NAD-rich media were able to adhere to lung alveoli of experimentally inoculated pigs. For the serotype 9 strain, this is in contrast with the results of previously described *in vitro* adhesion tests (Van Overbeke et al., 2002). For this strain, growth on NAD restricted media resulted in significantly more bacteria adhering to alveolar epithelial cells *in vitro* than when bacteria were grown on NAD rich media. The reason for this difference between *in vivo* and *in vitro* is not clear. Surfaces of *in vitro* cells however, may present proteins different from *in vivo* cells (Arp, 1988). Moreover, the surfactant layer covering the alveolar epithelial cells *in vivo* is absent when performing *in vitro* adhesion studies. Our finding that bacterial growth conditions did not influence the interactions of *A. pleuropneumoniae* with surfactant proteins also indicates that the adhesion of *A. pleuropneumoniae* to alveoli of pigs observed in the present studies, might be due to interactions with surfactant.

The *E. coli* strain did not adhere to alveolar epithelial cells. This suggests that the adhesion observed with the *A. pleuropneumoniae* strains was a specific interaction.

It remains to be determined which bacterial antigens are involved in binding of *A. pleuropneumoniae* to surfactant proteins. Several putative adhesins have already been described, including lipopolysaccharides that seem to be responsible for *in vitro* adhesion to porcine tracheal rings (Bélanger et al., 1990) and mucus (Bélanger et al., 1992&1994), a 80 kDa outer membrane protein that could play a role in the adherence of *A. pleuropneumoniae* to type III swine-lung collagen (Enriquez et al., 1999), a 55 kDa outer membrane protein and type IV fimbriae (Van Overbeke et al., 2002; Boekema et al., 2003). It is unlikely that the latter two antigens are responsible for binding of surfactant proteins since in the serotype 9 strain used in the present studies they are mainly expressed when bacteria are grown on a NAD restricted medium (Van Overbeke et al., 2002).

It is possible that more than one adhesin is involved in adhesion of *A. pleuropneumoniae* to alveoli of infected pigs. This already has been shown for related bacteria causing respiratory tract infections such as *Mannheimia haemolytica* (Brogden et al., 1989) and *Haemophilus influenzae* (Read et al., 1992). Association of *A. pleuropneumoniae* with surfactant might be a first step in colonization of alveoli followed by adhesion to the plasma membrane of alveolar epithelial cells. Such a two step adhesion has already been demonstrated for other bacteria including enterohemorrhagic *Escherichia coli* (Hicks et al., 1998).

To establish the role of different antigens in colonisation of alveoli further *in vivo* and *in vitro* studies with mutant strains lacking the capacity to express these candidate adhesins, are required.

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CHAPTER 3

EVALUATION OF THE EFFICACY OF A VACCINE CONTAINING CANDIDATE-ADHESINS

Effect of endobronchial challenge with *Actinobacillus pleuropneumoniae* serotype 10 of pigs vaccinated with bacterins consisting of *Actinobacillus pleuropneumoniae* serotype 10 grown under NAD-rich and NAD-restricted conditions

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SUMMARY

The efficacy of two bacterins containing an *Actinobacillus pleuropneumoniae* serotype 10 strain was evaluated. The bacterial cells constituting bacterin 1 and 2 were grown under NAD-rich (low adherence capacity to alveolar epithelial cell cultures) and NAD-restricted (high adherence capacity to alveolar epithelial cell cultures) conditions, respectively. Ten pigs were vaccinated twice with the bacterin 1 and 9 pigs with the bacterin 2. Ten control animals were injected twice with a saline solution. Three weeks after the second vaccination, all pigs were endobronchially inoculated with $10^{6.5}$ colony-forming units (CFU) of an *A. pleuropneumoniae* serotype 10 strain. In the bacterin 1 and 2 group, three and two pigs died after inoculation, respectively. Only two pigs of the control group survived challenge. Surviving pigs were killed at 7 days after challenge. The percentage of pigs with severe lung lesions (>10% of the lung affected) was 100% in the control group, 70% in the bacterin 1 group and 22% in the bacterin 2 group. *A. pleuropneumoniae* was isolated from the lungs of all animals. The mean bacterial titres of the caudal lung lobes were 7.0×10^6 CFU/g in the control group, 6.3×10^5 CFU/g in the bacterin 1 group and 1.3×10^6 CFU/g in the bacterin 2 group. It was concluded that both bacterins induced partial protection against severe challenge. Furthermore, there are indications that the bacterin 2, containing *A. pleuropneumoniae* bacteria grown under conditions resulting in high *in vitro* adhesion, induced better protection than the bacterin 1.

INTRODUCTION

Actinobacillus pleuropneumoniae causes porcine contagious pleuropneumonia, which is distributed world wide and results in serious losses in the pig rearing industry. It also causes severe animal suffering. Infected pigs may develop acute hemorrhagic necrotizing pneumonia and fibrinous pleuritis or chronic localized lung lesions and adhesive pleuritis (Taylor, 1999). To control outbreaks of this disease, vaccination may be useful. Vaccines containing the *A. pleuropneumoniae* Apx toxins have become commercially available and in previous studies, partial protection against endobronchial challenge in animals vaccinated with these vaccines was demonstrated (Chiers et al., 1998; Van Overbeke et al., 2001). Field trials carried out in France (Pommier et al., 1996), the Netherlands (Valks et al., 1996), Italy (Martelli et al., 1996), Spain (López et al., 1998) and Norway (Lium et al., 1998) confirmed that vaccination with Apx-containing vaccines can result in reduction of clinical symptoms and lung lesions of acute and chronic pleuropneumonia and improvement of performance (growth, feed conversion, cost of medication).

Apx toxins are not the only antigens that could play a role in protection against porcine pleuropneumonia. Capsular antigens, cell wall lipopolysaccharides, outer membrane proteins, fimbriae, transferrin binding proteins and adhesins also are important virulence factors (Haesebrouck et al., 1997).

Adherence to host tissue is generally regarded as an important prerequisite for colonization and virulence manifestation of bacteria (Ofek and Beachy, 1980).

In previous studies, it was shown that *A. pleuropneumoniae* adheres to alveolar epithelial cells of experimentally infected pigs (Dom et al., 1994a; 1995). It has also been shown that *A. pleuropneumoniae* adheres to porcine tracheal rings maintained in culture (Bélanger et al., 1990), to porcine frozen tracheal and lung sections Jacques et al., 1991), to erythrocytes from various animal species (Jacques et al., 1988), to porcine tonsillar epithelial cells (Chiers et al., 1999), to swine buccal epithelial cells (Hamer et al., 1999), to type III swine-lung collagen (Enriqueze et al., 1999) and to a culture of alveolar epithelial cells (Van Overbeke et al., 2002). In the latter study, it has been demonstrated that adhesive qualities of *A. pleuropneumoniae* to alveolar epithelial cells were influenced by growth conditions. Growth of *A. pleuropneumoniae* under NAD-restricted conditions results in more bacteria adhering to alveolar epithelial cells, indicating that a higher percentage of the bacterial population expresses adhesins under these conditions. The high adhesion scores of bacteria grown under NAD-restricted conditions have been related to expression of fimbriae and a 55 kDa outer membrane protein.

The present study was undertaken to determine if pigs, vaccinated with a bacterin consisting of bacteria grown under NAD-restricted conditions (high *in vitro* adherence capacity), were better protected or not against an *A. pleuropneumoniae* infection than pigs vaccinated with a bacterin consisting of bacteria grown under NAD-rich conditions (low *in vitro* adherence capacity).

MATERIALS AND METHODS

Challenge strain

An *A. pleuropneumoniae* serotype 10 reference strain D13039 was used. Bacteria were grown for 6 hours (log phase of growth) on Columbia agar (Columbia Agar Base, Lab M, Bury, Great Britain) supplemented with 3% horse serum, 0.03% NAD (Sigma Chemical Co, St Louis, Mo, USA), and 5% yeast extract at 37°C in a humid atmosphere with 5% CO₂. Bacteria were harvested in phosphate-buffered saline solution (PBSS, pH 7.3), centrifuged at 400 x g for 20 minutes, and suspended in RPMI (Roswell Park Memorial Institute) 1640 supplemented with 10% non-essential amino acids, 10% glutamine, 10% fetal calf serum, and 1% sodium pyruvate (Dom et al., 1994a). The suspension was checked for purity and the number of colony-forming units (CFU) was determined by plating tenfold dilutions on Columbia agar supplemented with 3% horse serum, 0.03% NAD, and 5% yeast extract. Bacterial suspensions were stored overnight at 4°C. The next day, they were used in the challenge experiments.

Pigs

Twenty-nine conventional pigs were obtained by using a medicated segregated early weaning programme (Chiers et al., 1998). They were weaned at 18 days of age and kept in isolation until used in the experiments.

Vaccine

Two bacterins were used. Bacterin 1 contained *A. pleuropneumoniae* serotype 10 strain D13039 grown on Columbia agar supplemented with 3% horse serum, 0.03% NAD and 5% yeast-extract for 6 hours at 37°C (NAD-rich medium). Bacterin 2 contained the *A. pleuropneumoniae* serotype 10 strain D13039 grown on Columbia agar supplemented with 1% horse serum and 0.001% NAD for 20 hours at 37°C (NAD-restricted medium).

The bacteria were harvested from the agar in PBSS, washed once with PBSS and resuspended in PBSS to achieve a suspension of 10⁹ CFU/ml. Bacteria in each suspension were inactivated by UV-light. This inactivation procedure does not affect *in vitro* adhesion to alveolar epithelial cell cultures (data not shown). After inactivation, the bacterins were emulsified in an equal volume of a mineral oil adjuvans (De Herdt et al., 1999).

Experimental design

At the age of 20 and 23 weeks, pigs were intramuscularly injected: 10 pigs with 2 ml of the bacterin 1, 9 pigs with 2 ml of the bacterin 2 and 10 pigs with 2 ml of a saline solution. Three weeks after the second injection, all pigs were experimentally infected. The pigs were anaesthetized with Zoletil® 100 (2.2 mg/kg tiletamine and 2.2 mg/kg zolazepam, Virbac, Louvain La Neuve, Belgium) and Xyl-M® 2% (4.4 mg/kg xylazine, VMD, Arendonk, Belgium).

They were inoculated endobronchially with $10^{6.5}$ CFU of the challenge strain in 5 ml inoculum (Van Overbeke et al., 2001). All pigs were examined clinically. Pigs that died were autopsied immediately; those that survived the challenge were killed 7 days after inoculation. At necropsy, the lungs were examined macroscopically and the percentage of affected lung tissue was determined. For this purpose, a diagram was used that divides the lung into 74 equal triangles (Hannan et al., 1982). The percentage of affected lung tissue was determined by summing up the triangles showing pneumonia and/or pleuritis divided by 74. Samples from lungs and tracheobronchial lymph nodes, were taken for bacteriological examination. Sera were collected before the first and the second vaccination and before the challenge.

Clinical examination

The pigs were examined for signs of pneumonia, characterized by increased respiratory rate (>40 inspirations/min), dyspnoea, sneezing, coughing, and the presence of a bloody foam on mouth and/or nostrils. Rectal temperature was measured 1 day before challenge inoculation, just before inoculation, and during the 7 days after inoculation. Other indications of infection were vomiting and a depressed appearance.

Bacteriological examination

Twenty per cent (w/v) suspensions of the right and the left caudal lung lobes were made in PBSS. The samples were tested for the presence of *A. pleuropneumoniae* by inoculating the suspensions onto Columbia agar supplemented with 5% bovine blood with a *Staphylococcus intermedius* streak. The number of cfu was determined by plating tenfold dilutions of the suspensions on Columbia agar supplemented with 3% equine serum, 0.03% NAD and 5% yeast extract. Samples from tracheobronchial lymph nodes were tested for the presence of *A. pleuropneumoniae* by making an incision in these tissues and taking a sample with an inoculation loop. These samples were inoculated onto Columbia agar supplemented with 5% bovine blood with a *Staphylococcus intermedius* streak. Identification of *A. pleuropneumoniae* was based on the CAMP reaction and the enhanced growth around the *Staphylococcus intermedius* streak.

Serology

All pig sera were tested for presence of ApxI neutralizing antibodies using a bioassay based on neutral red uptake by viable pulmonary alveolar macrophages, as described previously (Dom et al., 1994b) and for the presence of antibodies against cell-surface antigens of *A. pleuropneumoniae* serotype 10 strain D13039 grown on the NAD-rich and NAD-restricted media using an ELISA assay. The bacteria were harvested from the agar in PBSS, washed once with PBSS and resuspended in PBSS to achieve a suspension of 10^9 CFU/ml. After inactivation of the bacteria by UV-light, the suspension was centrifuged and resuspended in coatingbuffer (0.05M carbonate-bicarbonate buffer, pH 9.6) to achieve a suspension of 10^7

CFU/ml. Wells of a microtiter plate (Nunc-Immuno Plate MaxiSorp™, Gibco, Ghent, Belgium) were coated with 150 µl coatingbuffer containing the bacteria and incubated overnight at 4 °C. The next day, plates were washed four times with PBSS containing 0.05% Tween-20 (VWR International, Leuven, Belgium) (PBSS-T, pH 7.2). Non-specific binding was blocked by incubating the plates with 5% (w/v) glycine (VWR International) in PBSS-T. Plates were washed twice with PBSS-T, and were incubated with 1/200 dilution of sera. Bound antibodies were detected using 1/800 diluted peroxidase-conjugated rabbit anti-pig immunoglobulin serum (Sigma). Aminosalicilic acid and H₂O₂ (Sigma) were used as enzyme substrate. Reactions were read at 405 nm using a microELISA reader (Titertek Multiscan Plus). The OD values of wells devoided of sera served as background values.

Sera collected at the time of challenge were also tested for the presence of antibodies against a 55 kDa outer membrane protein of *A. pleuropneumoniae* serotype 10 strain D13039. Sarcosyl-insoluble outer membrane proteins of the *A. pleuropneumoniae* serotype 10 strain grown on the NAD-restricted medium were prepared as previously described (Van Overbeke et al., 2002). The outer membrane proteins were separated using discontinuous sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE). Electrophoresis experiments were performed with a 4.5% stacking gel and a 12% running gel. The antigen preparation was mixed with equal volumes of solubilization buffer [4% (w/v) of SDS, 10% (v/v) 2-mercapto ethanol, 17.4% (v/v) glycerol and 0.01% bromophenol blue in 0.06M Tris HCl (pH 6.8)] and boiled for 5 minutes prior to electrophoresis. After electrophoresis, antigens were electroblotted onto an immobilon polyvinylidene difluoride membrane (Millipore, Bedford, Mass., USA) using a semi-dry blotting system (190 mA, 1h, Bio-Rad Laboratories, Richmond, USA). Blotted proteins were subsequently probed with 1/100 diluted pig sera and 1/800 diluted anti-pig IgG serum conjugated with peroxidase (Sigma). Recognised antigens were visualised with chloronaftol and H₂O₂. To stop the reactions, the strips were washed three times in distilled water. A standard solution of prestained proteins with a molecular range from 14.2-97 kDa was used as a marker (Sigma, SDS-6B).

Statistical analysis

Statistical analysis was performed on the following variables: percentage of mortality, percentage of animals with fever, percentage of animals with dyspnoea, percentage of pigs with severe lung lesions (>10% of the lung affected), mean percentage of affected lung tissue and logarithmic mean of bacterial titre in caudal lung lobes.

A logistic regression analysis was used to compare the percentage of mortality, percentage of animals with fever, percentage of animals with dyspnoea, mean percentage of affected lung tissue and percentage of pigs with severe lung lesions (>10% of the lung affected). A Wilcoxon signed rank test was used to compare the log mean bacterial titres in caudal lung lobes between groups.

RESULTS

Results are summarized in Table 1.

Clinical examination

None of the animals showed disease signs before challenge and at the time of challenge.

In the control group (n=10), 6 pigs died and 2 animals were euthanised within 48 h after inoculation. The two remaining pigs showed dyspnoea and fever on day 1. Thereafter, one pig had dyspnoea and occasional coughing for another day and fever ($>40^{\circ}\text{C}$) for 6 days. The other pig had an increased respiratory rate during 4 days after challenge.

In the bacterin 1 group (n=10), one pig was euthanised one day after challenge and 2 other animals were euthanised on day 2. Respiratory signs (dyspnoea, increased respiratory rate or coughing) and fever could be detected in five animals. The duration of these signs varied between one day and 6 days. Two pigs had only an increased respiratory rate during 2 and 4 days. Three of the animals were depressed for 1 to 2 days.

In the bacterin 2 group (n=9), two pigs showed very severe symptoms and were euthanised. Four animals developed respiratory signs and fever. Duration of these symptoms varied between 1 and 7 days. Two animals had fever without respiratory symptoms. One pig showed an increased respiratory rate for 1 day. Two animals were depressed for 2 days.

A significant difference ($p<0.05$) was found for the percentage of mortality between the control group and the bacterin 1 group (low adhesin expression) ($p=0.033$) and bacterin 2 (high adhesin expression) group ($p=0.009$). There was also a significant difference for the percentage of animals with dyspnoea between the control group and the bacterin 2 (high adhesin expression) group ($p=0.049$).

Necropsy findings

In all animals lung lesions, when present, were characterized by a haemorrhagic necrotizing pneumonia and fibrinous pleuritis.

All animals of the control group had severe lung lesions ($>10\%$ of the lungs affected). The mean percentage of affected lung tissue was 56% (variation 16-100%).

In the bacterin 1 group, seven animals had severe lung lesions. One animal had very mild lung lesions and 2 animals had no lung lesions. The percentage of affected lung tissue varied between 0% and 57% (mean: 25%).

In the bacterin 2 group, two animals had severe lung lesions. Six animals had very mild lung lesions and 1 animal had no lung lesions. The percentage of affected lung tissue varied between 0% and 88% (mean: 16%).

A significant difference was found for the percentage of animals with severe lung lesions between the bacterin 2 (high adhesin expression) group and the control group ($p=0.007$) and between the bacterin 2 (high adhesin expression) group and the bacterin 1 (low adhesin expression) group ($p=0.047$). The mean percentage of affected lung tissue differed significantly between both bacterin groups and the control group ($p<0.001$) and between the

bacterin 2 (high adhesin expression) group and the bacterin 1 (low adhesin expression) group ($p < 0.001$).

Bacteriology

The results are summarized in Table 1.

In the control pigs, *A. pleuropneumoniae* was isolated from all lung lobes. The number of CFU/gram lung tissue of the caudal lobes varied between 3.0×10^5 and 2.8×10^8 , with a logarithmic mean of 7.0×10^6 . The challenge strain was isolated from the tracheobronchial lymphnodes of 8 pigs.

In the bacterin 1 group, *A. pleuropneumoniae* was isolated from all lung lobes. The number of CFU/gram lung tissue of the caudal lobes varied between 5.2×10^2 and 1.8×10^8 , with a logarithmic mean of 6.3×10^5 . *A. pleuropneumoniae* was isolated from the tracheobronchial lymphnodes of 1 pig.

In the bacterin 2 group, *A. pleuropneumoniae* was isolated from all lung lobes. The number of CFU/gram lung tissue of the caudal lobes varied between 3.6×10^4 and 9.4×10^7 , with a logarithmic mean of 1.3×10^6 . *A. pleuropneumoniae* was isolated from the tracheobronchial lymphnodes of 2 pigs.

No significant difference ($p < 0.05$) was found between the logarithmic mean of bacterial titres in the caudal lung lobes for the different groups.

Serology

In none of the animals, neutralizing antibodies against the Apx I toxin were detected.

Antibodies against cell-surface antigens were detected in both bacterin groups. OD values were in the same range for both antigen preparations. At the time of first vaccination, a mean OD value (standard deviation) was found of 0.132 (0.050) for the bacterin 1 group, 0.121 (0.059) for the bacterin 2 group and 0.124 (0.041) for the control group. At the time of second vaccination, a mean OD value (standard deviation) was found of 0.410 (0.103) for the bacterin 1 group, 0.408 (0.138) for the bacterin 2 group and 0.113 (0.23) for the control group. At the time of challenge, a mean OD value (standard deviation) was found of 0.454 (0.103) for the bacterin 1 group, 0.509 (0.161) for the bacterin 2 group and 0.132 (0.029) for the control group. The background OD values were in the same range as the OD values of the control group.

Antibodies against a 55 kDa outer membrane protein could not be detected in the sera collected at time of challenge.

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Table 1: Results of endobronchial challenge with *A. pleuropneumoniae* serotype 10 in pigs vaccinated with bacterin 1 and bacterin 2 and in non-vaccinated control pigs

	Bacterin 1 (low adhesin expression) (n=10)	Bacterin 2 (high adhesin expression) (n=9)	Controls (n=10)
% mortality	30 (a)	22 (a)	80 (b, c)
% animals with dyspnoea ¹	71	38 (a)	100 (c)
% animals with fever ^{1,2}	71	88	100
% animals with severe lung lesions (>10% of the lung affected)	70 (c)	22 (a, b)	100 (c)
mean % affected lung tissue	25 (a, c)	16 (a, b)	56 (b, c)
Logarithmic mean bacterial titre in caudal lung lobes in CFU/ml (standard deviation)	6.3×10^5 ($\pm 4.3 \times 10^1$)	1.3×10^6 ($\pm 1.1 \times 10^1$)	7×10^6 ($\pm 8.3 \times 10^0$)

1. only those pigs that survived challenge

2. fever is defined as a body temperature of $\geq 40^\circ\text{C}$

(a) = significant difference with control group

(b) = significant difference with bacterin 1 (NAD-rich) group

(c) = significant difference with bacterin 2 (NAD-restricted) group

DISCUSSION

Intranasal inoculation or aerosol exposure closely resemble the natural infection route of *A. pleuropneumoniae*. However, these methods are difficult to standardize because coughing, sneezing, swallowing, breathing and mucociliary clearance can reduce the number of bacteria that reach the alveolar region. In the present study, an endobronchial challenge model was used. An advantage of this model is that it delivers an exact number of bacteria into the bronchi, allowing standardization of experimental infections. Furthermore, this method allows us to bring the bacteria directly close to the multiplication site of *A. pleuropneumoniae*, namely the alveoli and bronchioli (Haesebrouck et al., 1997). A possible disadvantage is that the challenge strain is applied not all over the lung but is given more focal. Endobronchial challenge is therefore more likely to break through low level immunity in the respiratory tract than a more natural challenge route.

Eight of the 10 control animals died after infection. This demonstrates that a severe challenge model was used.

There was a significant difference in the percentage of mortality and the mean percentage of affected lung tissue between the control group and the bacterin groups. This indicates that both bacterins induced partial protection against severe challenge.

The percentage of animals with severe lung lesions and the mean percentage of affected lung tissue were significant lower in the bacterin 2 (high adhesin expression) group than in the bacterin 1 (low adhesin expression) group. Furthermore, there was also a significant difference for the percentage of animals with dyspnoea between the control group and the bacterin 2 (high adhesin expression) group. This could indicate that the bacterin 2, i.e. a bacterin containing bacteria grown under NAD-restricted conditions resulting in high *in vitro* adhesion to a culture of alveolar epithelial cells, provides better protection. Further studies are necessary to determine which antigens could be involved.

Bacterins were based on UV-inactivated and washed *A. pleuropneumoniae* bacteria. In sera of vaccinated animals, antibodies against the Apx I toxin were not found, suggesting that this toxin is not cell-associated but is only secreted into the culture medium. This is also mentioned by Frey and Nicolet (1991), but is in contrast with the reports by Fedorka-Cray (1989; 1993).

In previous studies, it was demonstrated that a 55 kDa outer membrane protein was expressed by the serotype 10 strain used in the present studies when bacteria were grown under NAD-restricted conditions (Van Overbeke et al., 2002). However, antibodies against this protein were not detected by Western blot analysis in sera of vaccinated animals at the time of challenge. The reason for this finding is not clear. It is possible that the immunogenicity of the 55 kDa outer membrane protein is low or that antibodies do not recognise the denaturated protein used in Western Blot analysis. Antibody reactions occurred between other outer membrane protein components and sera of vaccinated animals at the time of challenge (data not shown). However, there was no marked difference between the groups.

In the present studies, animals were only challenged with a serotype 10 strain. It has been shown that whole-cell bacterins based on bacteria grown in NAD-rich conditions generally do not confer protection against challenge with heterologous serotypes (Fenwick and Henry, 1994). An explanation for the limited protection might be the absence of secreted and certain bacteria-associated virulence factors in these bacterins. Further challenge studies with heterologous serotypes are necessary to determine if a vaccine containing bacteria grown in NAD-restricted conditions induces protection against other serotypes.

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GENERAL DISCUSSION

A. pleuropneumoniae causes severe losses in the pig rearing industry (Nicolet et al., 1992). Although the disease can be controlled by antimicrobial agents, the use of these products has several disadvantages including induction of acquired resistance in pathogenic bacteria and bacteria belonging to the normal flora of pigs. The latter bacteria may act as a reservoir of antimicrobial resistance genes for bacteria that cause disease in humans or animals. Excessive use of antimicrobials may also result in the presence of residues in meat. Therefore, prevention is a better option for the control of the disease. Vaccines could be very useful to control porcine pleuropneumonia. Rational design of effective antibacterial vaccines, however, requires knowledge of the virulence factors of the bacterium and the pathogenesis of the disease. Pathogenesis of porcine pleuropneumonia is very complex and several virulence factors have been described. Some of these virulence factors, such as the Apx toxins, have been extensively studied. Virulence attributes involved in lung colonisation are, however, less understood. The need for research in the first steps of pathogenesis provided the main rationale for this thesis.

Recovery from naturally acquired infection with *A. pleuropneumoniae* provides solid, serotype cross-protective immunity. Evidence from field and experimental studies indicates that infection with one serotype of *A. pleuropneumoniae* provides complete protection against subsequent infection with the homologous serotype and at least partial protection against heterologous infection (Nielsen et al., 1984; Haesebrouck et al., 1996). Preferably, protection induced by vaccines should approach protection induced by natural infection.

The first vaccines that were developed against porcine pleuropneumonia were whole-cell bacterins, consisting of inactivated bacterial cell suspensions and supplemented with an adjuvant. Although these vaccines have the advantage of presenting a complex array of antigenic determinants to the immune system, inactivation procedures may alter the antigenic patterns of a bacterin and may be responsible for the loss of relevant immunogenic epitopes. This can be overcome by using ghost vaccines. Bacterial ghosts are empty cell envelopes achieved by the expression of a cloned bacteriophage lysis gene and, unlike classical bacterins, suffer no denaturing steps during their production. These properties may lead to a superior presentation of surface antigens to the immune system (Hensel et al., 2000). Whole-cell bacterins may reduce mortality after infection with the homologous serotype, but they generally do not confer protection against challenge with heterologous serotypes. An explanation for the limited protection might be the absence of Apx toxins. Apx toxins are indeed thought to be of particular importance for the induction of protective immunity. Frey and Nicolet (1991) reported that Apx toxins are secreted by the bacterium in the culture medium and that they are not cell-associated. However, this is in contrast with reports by Fedorka-Cray (1989; 1993). Results of our studies also suggest that the Apx toxins are not

cell-associated since antibodies against Apx toxins were not detected in sera of animals vaccinated with bacterins based on UV-inactivated and washed *A. pleuropneumoniae* bacteria.

Improved protection has been obtained by using subunit vaccines containing the Apx toxins. Among the different serotypes of *A. pleuropneumoniae*, 4 of these exotoxins have been described. In contrast to ApxI, ApxII and ApxIII toxins, each of which is produced by some but not all serotypes, the ApxIV toxin is produced by all serotypes (Cho and Chae, 2001; Schaller et al., 1999). Apparently, the ApxIV toxin is only produced *in vivo* (Schaller et al., 1999). Therefore, the current commercially available vaccines do not contain this ApxIV toxin. Results of the present thesis and field trials demonstrated that vaccination with vaccines containing ApxI, ApxII and ApxIII can result in a reduction of the clinical symptoms and lung lesions of acute and chronic pleuropneumonia and improvement of performance (growth, feed conversion, cost of medication) (Pommier et al., 1996; Valks et al., 1996; Martelli et al., 1996; Lopez et al., 1998; Lium et al., 1998).

Keeping in mind the very complex pathogenesis of porcine pleuropneumonia, inclusion of other bacterial virulence factors in subunit vaccines might be of value. Results of studies described in this thesis demonstrated that a vaccine containing both Apx toxins and transferrin binding proteins induced better protection against severe challenge than a vaccine solely based on Apx toxins. Recently, it was shown that antibodies induced against purified PalA, the most immuno-predominant outer membrane protein of *A. pleuropneumoniae*, aggravated the consequences of an experimental infection and counteracted the protective effect of anti-Apx antibodies (van den Bosch and Frey, 2003). Therefore, care should be taken which antigens are included in subunit vaccines. In whole-cell preparations of bacterial cultures, the concentration of PalA may vary depending on the mode of cultivation and preparation of the bacteria. This may be an explanation for the variation in protective efficacy of different bacterins.

Bacterial virulence factors that, most probably, would be very valuable to include in vaccines are adhesins. Indeed, *A. pleuropneumoniae* first adheres to alveolar epithelial cells before it causes lung lesions (Dom et al., 1992). Antibodies that interfere with adhesion could block this first step in the pathogenesis of porcine pleuropneumonia and protect pigs against lung infection. In chapter 3 of this thesis it was shown that a vaccine containing inactivated *A. pleuropneumoniae* bacteria grown under circumstances resulting in high *in vitro* adhesion induced better protection than a vaccine containing inactivated *A. pleuropneumoniae* bacteria grown under circumstances resulting in low *in vitro* adhesion.

When we started our studies, no reliable adherence model was available to study the way *A. pleuropneumoniae* interacts with alveolar epithelial cells. The *in vitro* adherence model developed during these studies enabled us to investigate the first interactions between *A. pleuropneumoniae* and the alveolar epithelial cells. We were also able to pinpoint the adherence mechanisms and to characterize some candidate-adhesins.

There are different methods for studying bacterial adherence to tissue culture cells: (1) Colony count: the cell layer can be lysed with an appropriate detergent which does not affect bacterial viability. Appropriate dilutions are then plated on agar plates for enumeration of cell-associated bacteria by colony count (Robbins-Browne and Bennett-Wood, 1992). The number of cell-associated bacteria is most commonly expressed as a percentage of the original bacterial inoculum. (2) Radioactivity count: bacteria can be labelled with a radioactive isotope before investigation by the adhesion assay. The number of cell-associated bacteria can then be measured by lysing the cell monolayer, and measuring the radioactivity of the lysate (Dunkle et al., 1986). (3) Enzyme linked immunosorbent assay: the number of cell-associated bacteria can be quantitated by ELISA, by either using specific antibodies to the adherent bacteria followed by enzyme-conjugated secondary antibody, or by using biotinylated bacteria and avidin-enzyme as the detecting agent (Ofek et al., 1986). (4) Fluorescence flow cytometry, (5) Light and electron microscopy as was used in our studies. Microscopic examination has several advantages. It allows us to determine the pattern of adherence of bacteria and the proportion of host cells with adherent bacteria. Also, an estimation of the number of bacteria adherent to each host cell can be made and changes in cell morphology as a result of bacterial adherence can be examined (Nataro et al., 1987; Zafiri et al., 1989).

Cultured cells provide a simple and easily controlled model for investigating the host-bacterium interaction. Cells can be grown under reproducible conditions and only one cell type is represented. Due to the variation in expression of cell surface epitopes known to exist between species and tissues/organs, as well as among cells originating from the same tissue as a function of developmental and differentiation stages (Falk et al., 1994a; Falk et al., 1994b), careful consideration must be made to the type of cultured cell selected for the adhesion assays. The cultured cell chosen in these studies, i.e. the alveolar epithelial cell, is the naturally colonized cell with which the organism interacts.

The *in vitro* adhesion model offers a valuable alternative for the use of live animals, in line with the Three Rs concept (replacement, reduction and refinement) of Russell & Burch (1959). Any experimental system which does not entail the use of a whole, living animal is considered to be a replacement alternative. The *in vitro* adhesion model developed here is a relative replacement, as it still entails the humane killing of an animal for the purpose of obtaining cells for subsequent *in vitro* studies. Although animals are needed to collect the cells, the animals are used more economically, because a single animal provides cells for a number of cultures. To collect the alveolar epithelial cells, pain and suffering was minimised as the animals were anaesthetised and euthanised prior to the collection of the cells. In that way, the animals merely act as organ donors and are not subjected to painful infectivity studies.

However, there are also disadvantages to the use of *in vitro* models. In the *in vivo* situation, the interplay between the alveolar epithelial cells and *A. pleuropneumoniae* occurs at an air interface. Tsang et al. (1994) have described a novel tissue culture model to study the interaction of *Pseudomonas aeruginosa* with adenoid tissue in which bacterial interaction with respiratory mucosa occurs in air-mucosal interphase. Evenso, Jackson et al. (1996) compared the interaction of *Haemophilus influenzae* type b with adenoid tissue cultures maintained with an air interface or immersed in medium. They observed that bacterial adhesion to mucus and damaged epithelium was significantly higher in the mucosa-air interface model than in immersed tissue cultures. They concluded that immersion of respiratory tissue during experimental infection can substantially influence the results obtained. Moreover, the surfactant layer covering the alveolar epithelial cells *in vivo* is absent in the *in vitro* adhesion model. Therefore, data obtained from *in vitro* experimental models should be confirmed in animal models.

In the *in vitro* adhesion assay, porcine alveolar epithelial cells were allowed to attach to coverslips whereafter they were fixed with methanol. Fixation was used to prevent destruction of the alveolar epithelial cells by the Apx toxins of *A. pleuropneumoniae* (Van de Kerkhof et al., 1996). Another method to prevent cytotoxic effects is fixing the cells in formalin (Spencer et al., 1997). Preliminary studies indicated that *A. pleuropneumoniae* does not adhere to formalin fixed alveolar epithelial cells, most probably because this results in an alteration or destruction of receptors on the host cells.

In our *in vitro* adhesion studies, strains belonging to the biotype 1-serotypes 2, 5a, 9 and 10 were used. These serotypes were selected because they are commonly found in Belgium (Hommeze et al., 1988; Hommeze et al., 1990; Dom et al., 1994).

Important considerations before performing bacterial adhesion assays are the bacterial growth conditions, as they will influence the expression of virulence factors during the course of infection. In general, growth conditions approximating the *in vivo* environment have been found to produce the best results. *A. pleuropneumoniae* biotype 1 is NAD-dependent. We postulated that extracellular NAD-concentrations in the lumen of the lung alveoli, most probably are very low, since, based on molecular weight and charge, it is unlikely that leakage of NAD out of normal living cells occurs. This was confirmed by O'Reilly and Niven (2003). They determined the concentrations of NAD in the extracellular fluids of pigs, more precisely in plasma, tissue fluids, and laryngeal, tracheal and lung washings and found that the levels in these extracellular fluids were considerably lower than those found in human erythrocytes and in rat tissues.

Three different growth media were used: a medium containing 0.03% NAD which was defined as NAD-rich and two media containing 0.001% NAD which were defined as NAD-restricted. For the serotype 5a, 9 and 10 strains, optimal adherence was observed after growth of bacterial cells in a NAD-restricted medium (0.001% NAD). For the serotype 2 strain,

adherence was less influenced by the growth conditions. This indicates that expression of adhesins is strain or serotype-dependent.

Mechanisms by which bacteria maintain close proximity to a mucosal surface can be loosely categorized as association, adhesion, and invasion according to the degree of intimacy between bacterial and mucosal surfaces. The most intimate form of bacterial-mucosal interaction is invasion, wherein bacteria penetrate the mucosal barrier to establish themselves within epithelial cells or adjacent stromal tissue. Association, the least intimate form of surface interaction, implies weak, reversible attachment or localization of bacteria along a surface (Marschall, 1984). This may be the result of the combined effects of van der Waals forces, hydrogen bonds, and ionic and hydrophobic interactions (Jones, 1977). Adhesion, a more intimate form of attachment than association, describes relatively stable, irreversible attachment mediated by specialized complementary molecules in a ligand-receptor fashion with numerous noncovalent bonds between surfaces of bacteria and substratum (Jones and Isaacson, 1983). This interaction typically occurs between bacterial surface proteins and carbohydrate-containing molecules of the cell membrane or glycocalyx (Lark, 1986). An adhesin is any bacterial structure or molecule that mediates adhesion. Receptors are components of the mucosal surface which bind in a complementary fashion with the active site of the corresponding adhesin during specific bacterial adhesion.

In general, irreversible attachment of bacteria to mucosal surfaces is mediated by a variety of complex polymers on the bacterial surface. Results of chapter 2 demonstrate that proteins are involved in adhesion of *A. pleuropneumoniae* to alveolar epithelial cells as indicated by a decrease in adherence after pretreating the bacteria with heat, pepsin, trypsin, and protease. Proteinaceous bacterial adhesins can be divided into those with fimbrial morphology and those lacking definite size and shape.

Fimbriae and pili are morphologic terms used synonymously to denote nonflagellar, filamentous appendages that radiate from the bacterial surface. The filamentous structure of most fimbriae is readily apparent on electron microscopy of negatively stained bacterial suspensions (Jones and Isaacson, 1983). Fimbriae are arranged in a polar or peritrichous fashion on the bacterial cell and vary in number from a few to several hundred.

Surface protein adhesins that lack definitive morphology tend to be outer membrane proteins or secreted proteins that remain loosely associated with the bacterial surface. *Bordetella pertussis* produces secreted proteins involved in adhesion to cilia of the respiratory epithelium (Cowell et al., 1986). Adhesion of *Neisseria gonorrhoeae* to epithelial cells is mediated not only by fimbriae, but also by a group of closely related outer membrane proteins (Swanson, 1983). Outer membrane proteins are also involved in the adhesion of *A. actinomycetemcomitans* to epithelial cells (Jacques and Paradis, 1998) and in adhesion of *Pasteurella multocida* to respiratory mucosal surface preparations (Lübke et al., 1994). For *Mannheimia haemolytica*, it has been proposed that outer membrane proteins may function as adhesins in the absence of fimbriae (Confer et al., 1995).

Our studies indicate that both fimbriae and outer membrane proteins might be involved in adhesion of *A. pleuropneumoniae* to alveolar epithelial cells. Indeed, fimbriae and a 55 kDa outer membrane protein were mainly expressed when *A. pleuropneumoniae* strains were grown on media resulting in optimal adherence.

Proving that a putative adhesin causes the particular biological effect, i.e. adhesion, is not easy (Smith, 1983). The main method of strengthening the evidence for causation is to neutralize the adherence capacity of intact virulence organisms by specific antibody raised against the purified putative determinant. Antibody specificity is crucial. Monoclonal antibodies could be useful in this respect, but although they have been used extensively in identifying bacterial surface components, their use in proving causation of biological effect is limited (Sparling, 1983).

Another method is to perform adhesion tests with alveolar epithelial cells that were pre-incubated with the purified putative adhesins. For purification of fimbriae, these structures should first be removed from the bacterial surface. This can be obtained by mechanical disruption (Imberechts et al., 1992; Wu et al., 1995; Van Alphen et al., 1988) or by heat treatment (Yoshimatsu et al., 1991). Since the number of fimbriae observed on *A. pleuropneumoniae* cells is low, it can be expected that purification will not be easy. Indeed, in preliminary studies it was not possible to obtain sufficient amounts of fimbriae after mechanical disruption or heat treatment to allow purification (unpublished data).

DNA technology can also be very useful for proving causation and relevance for virulence determinants on bacterial surfaces.

One aspect of genetic manipulation is the use of mutants. Mutants lacking the putative virulence determinant are compared with the wild type in the appropriate biological test. Another aspect of genetic manipulation is the use of gene cloning. There are many cloning strategies, mostly invoking *E. coli*. The *E. coli* clone can be used to demonstrate directly the biological activity of the putative determinant and can also produce it. More importantly, the cloned gene can then be reintroduced into deficient strains of the original pathogen, thereby confirming the biological property and virulence *in vivo*.

The fimbriae observed on *A. pleuropneumoniae* have been identified as type 4 fimbriae (Zhang et al., 2000). This is according to the classification scheme by Duguid et al. (1966). Type 1 refers to fimbriae that are rigid and exhibit mannose-sensitive hemagglutination; type 2 is serologically related to type 1 but does not cause hemagglutination; type 3 refers to mannose-resistant, flexible fimbriae common in *Enterobacteriaceae*; type 4 are fimbriae possessing N-methylphenylalanine in the amino terminus region of the major subunit; and type 5 are mannose sensitive, thinner than type 1, and usually few in numbers per cell. Type 4 fimbriae have been described in several other Gram-negative bacteria including *Haemophilus influenzae*, *Moraxella bovis*, *Dichelobacter nodosus*, *Haemophilus ducreyi*, *Pasteurella multocida* and *Pseudomonas aeruginosa*. It has been shown that they mediate

attachment of these bacteria to epithelial cells *in vivo* and *in vitro* (Sato and Okinaga, 1987; Doig et al., 1988; Paranchyn and Frost, 1988; Starr et al., 1999).

Recently, Stevenson et al. (2003) reported the identification and characterization of the type 4 fimbrial structural gene (*apfA*) of *A. pleuropneumoniae*. In addition a number of open reading frames were identified that have significant homology to type 4 fimbrial biogenesis genes from other species. These type 4 fimbrial biogenesis genes showed the strongest homology to putative type 4 fimbrial genes of *Haemophilus ducreyi*. Recombinant fimbriae produced by expressing type 4 subunits from *Moraxella bovis* (Beard et al., 1990) and *Dichelobacter nodosus* (Elleman et al., 1986) in *Pseudomonas aeruginosa* have been used as vaccine antigens and this type of approach might be interesting for an *A. pleuropneumoniae* ApfA based vaccine. Furthermore, it was shown by Boekema et al. (2003) that contact with epithelial cells is a trigger for fimbriae production and that there is an *in vivo* induced activity of the fimbriae promoter shortly after infection, suggesting that fimbriae play a role in the colonisation and the pathogenesis.

Surface hydrophobicity of bacteria is an important physical factor for adhesion, especially when the substrata are either hydrophilic or hydrophobic. Generally, bacteria with hydrophobic properties prefer materials with hydrophobic surfaces, those with hydrophilic characteristics prefer hydrophilic surfaces (Hogt et al., 1983; Satou et al., 1988; Vacheethasanee et al., 1998). To assess the role of hydrophobic interactions in adherence, we studied the influence of the presence of the hydrophobic bond-breaking agent tetramethylurea. This technique was also used by Bélanger et al. (1994) in relation to *A. pleuropneumoniae* adherence to crude mucus. Hydrophobic interactions did not seem to participate in the binding of *A. pleuropneumoniae* to alveolar epithelial cells *in vitro* since tetramethylurea had no effect on binding.

Many adhesins act as lectins, recognizing specific carbohydrate moieties on host cell surfaces. Therefore, bacterial cells were subjected to treatments with various sugars to determine whether carbohydrate-specific lectins on the surface of *A. pleuropneumoniae* cells are involved in adherence. The various sugars we used were mono- and disaccharides. The finding that none of these sugars caused decreased adherence does not mean that *A. pleuropneumoniae* can not interact with specific carbohydrates in adherence processes. Indeed, adhesins can recognize complex carbohydrates as has been described for the P fimbriae of *Escherichia coli*. These fimbriae recognize complex carbohydrates such as globotriasoylceramide and globoside (Bock et al., 1985). Moreover, it can not be excluded that different types of lectins are present on the bacterial cells or that a heterogeneous population of bacterial cells is present, each bearing a different lectin (Ofek and Sharon, 1990). In such cases, a combination of sugars is a more effective inhibitor than individual ones. This, however, remains to be tested.

Treatment of *A. pleuropneumoniae* strains with sodium metaperiodate resulted in decreased adhesion of the bacteria to alveolar epithelial cells, but inhibition was clearly lower than after treatment of bacteria with proteolytic enzymes. The finding that inhibition of adhesion was very high when bacteria were treated with a combination of sodium metaperiodate and pronase suggests that more than one adhesin is involved. Indeed, although adhesion of bacteria to host cells may depend on the binding of a unique bacterial adhesin to a unique host receptor, most frequently bacterial adhesion depends on simultaneous interaction between multiple adhesins with different cell receptors. The inhibition of a single pathway therefore may have no effect on overall adhesion or at best may produce a partial inhibition. Most *Pasteurellaceae* organisms can employ multiple molecular mechanisms of adherence to initiate infection (Jacques and Paradis, 1998). Proteins (e.g. fimbriae, fibrils, outer membrane proteins) and/or polysaccharides were shown to play a role in adherence of several members of this bacterial family. These multiple adhesins may be used in a stepwise fashion during colonization. It seems reasonable that evolutionary pressures have selected organisms that are capable of using more than one mechanism of adherence (Ofek and Doyle, 1994).

As stated before, it is very important to confirm data obtained from *in vitro* models in animal models. This was certainly recommended in our studies because the surfactant layer covering the alveolar epithelial cells *in vivo* is absent in the *in vitro* adhesion model. Pulmonary surfactant is a lipoprotein complex that is synthesized and secreted by the alveolar type II epithelial cell and the airway Clara cell (Gourke, 1998). Once in the extracellular space, surfactant reduces surface tension at the air-liquid interface of the lung, a function that requires an appropriate mix of surfactant lipids and the hydrophobic proteins, surfactant protein B (SP-B) and SP-C (Whitsett, 1995). Phospholipids of several types are the major components of surfactant. Phospholipids are amphipathic molecules with a polar head group and a nonpolar tail. These hydrophobic tails are extended into the air. The single most abundant phospholipid is dipalmitoylphosphatidylcholine (Hawgood, 1991). Other phospholipids are: unsaturated phosphatidylcholines, phosphatidylglycerol, phosphatidylethanolamine and phosphatidylserine. It is possible that *A. pleuropneumoniae* interacts with surfactant as a first step in the colonization. We demonstrated that *A. pleuropneumoniae* binds to surfactant proteins. Moreover, *in vivo* adhesion studies resulted in adherence of *A. pleuropneumoniae* to lung alveoli irrespective of the growth conditions of the bacteria. This was in contrast with the *in vitro* adhesion studies whereby growth on NAD restricted media resulted in significantly higher adhesion scores in serotype 5, 9 and 10 strains. Hydrophobicity tests as described by Vercauteren et al. (1993) did not show differences in hydrophobicity between bacteria grown under different growth conditions (unpublished results). All these results indicate that adhesion of *A. pleuropneumoniae* to alveoli is a two step adhesion. A first step could be a reversible association of *A. pleuropneumoniae* with surfactant. Lipopolysaccharides might be involved in this association since Jeannotte et al. (2003) described that *A. pleuropneumoniae* can bind to

phosphatidylethanolamine. Such association has also been described for other pathogenic bacteria including *Chlamydia pneumoniae* and *Chlamydia trachomatis* (Krivan et al., 1991), *Helicobacter pylori* (Lingwood et al., 1992), *Helicobacter mustelae* (Gold et al. 1993), *Haemophilus influenzae* (Busse et al., 1997) and *Campylobacter upsaliensis* (Busse et al., 1997).

A second step could be an adhesion step to alveolar epithelial cells i.e. a relatively stable, irreversible attachment mediated by specialized complementary molecules in a ligand-receptor fashion. The above described candidate-adhesins i.e. fimbriae and a 55 kDa outer membrane protein could play a role in this adhesion.

Surfactant also plays a role in host defence against infection and inflammation (Wright, 1997&2003). Two of the surfactant proteins, SP-A and SP-D, are members of the collectin protein family (Crouch and Wright, 2001; Crouch et al., 2000). The most well-defined function of the collectins is their ability to opsonize pathogens, including bacteria and viruses, and to facilitate phagocytosis by innate immune cells such as macrophages and monocytes. It is unlikely that opsonization of *A. pleuropneumoniae* and subsequent phagocytosis by macrophages and monocytes will result in killing of *A. pleuropneumoniae*. Indeed, Cruikshank et al. (1992) demonstrated that porcine alveolar macrophages effectively phagocytise virulent strains of *A. pleuropneumoniae* in the presence of convalescent pig serum but they are not able to kill the bacteria intracellularly. This is most probably due to intracellular released Apx toxins (Cruikshank et al., 1992). Data presented by Wu and coworkers (2003) show convincingly that, in addition to facilitating pathogen uptake and killing by immune cells, SP-A and SP-D are directly antimicrobial, that is, they kill bacteria in the absence of immune effector cells. The effect of these surfactant proteins on *A. pleuropneumoniae* has not yet been studied.

In conclusion, results of the present thesis confirm that the currently available subunit vaccines, based on Apx toxins, induce partial protection. Inclusion of adhesins in such vaccines might improve efficacy. Adhesion of *A. pleuropneumoniae* to alveolar epithelial cells is a very complex phenomenon and several bacterial antigens seem to be involved including outer membrane proteins and fimbriae. Additional studies are necessary to further characterize virulence factors responsible for adhesion of *A. pleuropneumoniae* to host cells.

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SUMMARY

A. pleuropneumoniae causes severe losses in the pig rearing industry. Although the disease can be controlled by antimicrobial agents, the use of these products has several disadvantages including induction of acquired resistance in pathogenic bacteria and bacteria belonging to the normal flora of pigs. Therefore, prevention instead of therapy is an increasing requirement in the control of the disease. Vaccines could be very useful to control porcine pleuropneumonia. Rational design of effective antibacterial vaccines, however, requires knowledge of the virulence factors of the bacterium and the pathogenesis of the disease. Pathogenesis of porcine pleuropneumonia is very complex and several virulence factors have been described. Some of these virulence factors, such as the Apx toxins, have been extensively studied. Virulence attributes involved in lung colonisation are, however, less well known. The need for research in these first steps of pathogenesis provided the main rationale for this thesis.

The experiments performed in this thesis are divided in 3 Chapters. In Chapter 1, the efficacy of vaccines containing Apx toxins was evaluated. Thereafter, the adhesion of *A. pleuropneumoniae* to porcine alveolar epithelial cells *in vitro* and *in vivo* was studied (Chapter 2). Finally, the efficacy of a vaccine containing candidate-adhesins was evaluated (Chapter 3).

In a first study described in Chapter 1, the efficacy of two inactivated vaccines containing the Apx toxins of *Actinobacillus pleuropneumoniae* (Hemopig™, Biokema, Lausanne, Switzerland and Porcilis™ App, Intervet, Boxmeer, The Netherlands) was determined. Therefore, ten pigs were vaccinated twice with Hemopig™ and eight pigs with Porcilis™ App. Ten control animals were injected twice with a saline solution. Three weeks after the second vaccination, all pigs were endobronchially inoculated with 10^5 colony-forming units (CFU) of an *A. pleuropneumoniae* serotype 9 strain. Increased respiratory rate and/or fever were observed in all vaccinated and control pigs after challenge. One pig of the Hemopig™ group and of the Porcilis™ App group died, whereas all pigs of the control group survived the challenge. Surviving pigs were killed at 7 days after challenge. The mean percentage of affected lung tissue was 34% in the control group, 16% in the Hemopig™ group, and 17% in the Porcilis™ App group. *A. pleuropneumoniae* was isolated from the lungs of all 10 control animals, from 7 of the 10 animals vaccinated with Hemopig™ and from 5 of the 8 animals vaccinated with Porcilis™ App. The mean bacterial titres of the caudal lung lobes were 1.4×10^6 CFU/g in the control group, 1.7×10^3 CFU/g in the Hemopig™ group, and 4.8×10^3 CFU/g in the Porcilis™ App group. In both vaccinated groups the mean number of days with dyspnoea, the mean

number of days with fever, the mean percentage of affected lung tissue, and the mean bacterial titre in the caudal lung lobes were significantly lower than in the control group. Significant differences between the two vaccinated groups were not observed. It was concluded that both vaccines induced partial protection.

In a second study, the efficacy of a subunit vaccine containing the Apx toxins and transferrin binding proteins was determined. Therefore, ten pigs were vaccinated twice with the vaccine. Eight control animals were injected twice with a saline solution. Three weeks after the second vaccination, all pigs were endobronchially inoculated with $10^{6.5}$ colony-forming units (CFU) of an *A. pleuropneumoniae* serotype 9 strain. In the vaccine group, none of the pigs died after inoculation. Only one pig of the control group survived challenge. Surviving pigs were killed at 7 days after challenge. The mean percentage of affected lung tissue was 64% in the control group and 17% in the vaccine group. *A. pleuropneumoniae* was isolated from the lungs of all animals. The mean bacterial titres of the caudal lung lobes were 5.0×10^8 CFU/g in the control group and 3.0×10^6 CFU/g in the vaccine group. It was concluded that the vaccine induced partial protection against severe challenge.

Keeping in mind the very complex pathogenesis of porcine pleuropneumonia, inclusion of other bacterial virulence factors in subunit vaccines might be of value. Bacterial virulence factors that, most probably, would be very valuable to include in vaccines are adhesins. Indeed, *A. pleuropneumoniae* first adheres to alveolar epithelial cells before it causes lung lesions. Antibodies that interfere with adhesion could block this first step in the pathogenesis of porcine pleuropneumonia and protect pigs against lung infection. Therefore, the adhesion of *A. pleuropneumoniae* to cells *in vitro* and *in vivo* was studied (Chapter 2).

In a first study, the adhesion ability of *A. pleuropneumoniae* biovar 1 serotype 2, 5a, 9 and 10 strains was tested to alveolar epithelial cells in culture. For the serotype 5a, 9 and 10 strains, optimal adherence was observed after growth of bacterial cells in a NAD-restricted medium (0.001% NAD). This condition was also associated with the expression of a 55 kDa outer membrane protein and of fimbriae. For the serotype 2 strain, adherence and expression of fimbriae and a 55 kDa outer membrane protein was less influenced by the growth conditions. The N-terminal amino acid sequence of the 55 kDa outer membrane protein had no homology with any known sequence, suggesting that it is an as yet unknown protein. Adherence capabilities were significantly reduced following treatment of the bacteria with proteolytic enzymes or heat. These findings suggest that proteins are involved in adhesion. The hydrophobic bond breaking agent tetramethylurea was unable to inhibit the adherence of *A. pleuropneumoniae* to alveolar epithelial cells. Treatment of the bacteria with sodium metaperiodate resulted in lower adhesion scores for the serotype 2 and 9 strains but the inhibition of adhesion was clearly lower than after treatment with proteolytic enzymes. This indicates that, besides proteins, carbohydrates might also be involved in adhesion of *A. pleuropneumoniae* to alveolar epithelial cells. The finding that inhibition of adhesion was very

high when bacteria were treated with a combination of sodium metaperiodate and pronase also suggests that more than one adhesin is involved.

Secondly, the effect of culture conditions on adhesion of inactivated *A. pleuropneumoniae* biotype 1-serotype 2 and 9 strains to alveoli of experimentally inoculated piglets was studied. Growing the bacteria on a NAD-restricted or NAD-rich medium did not influence this adhesion and had no effect on the *in vitro* association of the bacteria with surfactant proteins. Adhesion scores in alveolar epithelial cell cultures were, however, much higher when the serotype 9 strain was grown on the NAD-restricted medium. It is possible that different adhesins are involved in colonisation of alveoli of infected pigs. Association with surfactant might be a first step that precedes adhesion to alveolar epithelial cells.

Finally, in Chapter 3, the efficacy of two bacterins containing an *A. pleuropneumoniae* serotype 10 strain was evaluated. The bacterial cells constituting bacterin 1 and 2 were grown under NAD-rich (low adherence capacity to alveolar epithelial cell cultures) and NAD-restricted (high adherence capacity to alveolar epithelial cell cultures) conditions, respectively. Ten pigs were vaccinated twice with the bacterin 1 and 9 pigs with the bacterin 2. Ten control animals were injected twice with a saline solution. Three weeks after the second vaccination, all pigs were endobronchially inoculated with $10^{6.5}$ colony-forming units (CFU) of an *A. pleuropneumoniae* serotype 10 strain. In the bacterin 1 and 2 group, three and two pigs died after inoculation, respectively. Only two pigs of the control group survived challenge. Surviving pigs were killed at 7 days after challenge. The percentage of pigs with severe lung lesions (>10% of the lung affected) was 100% in the control group, 70% in the bacterin 1 group and 22% in the bacterin 2 group. *A. pleuropneumoniae* was isolated from the lungs of all animals. The mean bacterial titres of the caudal lung lobes were 7.0×10^6 CFU/g in the control group, 6.3×10^5 CFU/g in the bacterin 1 group and 1.3×10^6 CFU/g in the bacterin 2 group. It was concluded that both bacterins induced partial protection against severe challenge. Furthermore, there are indications that the bacterin 2, containing *A. pleuropneumoniae* bacteria grown under conditions resulting in high *in vitro* adhesion, induced better protection than the bacterin 1.

SAMENVATTING

A. pleuropneumoniae veroorzaakt grote economische verliezen in de varkenshouderij. Bij de bestrijding van pleuropneumonie wordt vaak gebruik gemaakt van antibiotica. Alhoewel antibiotica hun nut bewezen hebben, zijn er aan het overvloedig gebruik ervan verschillende nadelen verbonden zoals het optreden van verworven resistentie. Het is duidelijk dat dient gezocht te worden naar alternatieve bestrijdingsstrategieën. Hierbij wordt in de eerste plaats gedacht aan vaccinatie. Bij de ontwikkeling van effectieve antibacteriële vaccins, is een grondige kennis van de virulentiefactoren van de kiem en de pathogenese van de ziekte noodzakelijk. De pathogenese van pleuropneumonie is zeer complex en er werden reeds verschillende virulentiefactoren beschreven. Sommige van deze virulentiefactoren, zoals de Apx toxines, werden in detail bestudeerd. Virulentiefactoren die betrokken zijn in de kolonisatie van de long zijn echter minder gekend. Onderzoek naar deze eerste stappen in de pathogenese was dan ook het hoofdobjectief van dit proefschrift.

Het experimentele gedeelte van dit proefschrift wordt in 3 hoofdstukken ingedeeld. Vooreerst werd de doeltreffendheid van vaccins die Apx toxines bevatten, bepaald. Daarna werd de adhesie van *A. pleuropneumoniae* aan alveolaire epitheelcellen *in vitro* en *in vivo* bestudeerd (Hoofdstuk 2). Tenslotte werd de doeltreffendheid bepaald van een vaccin dat kandidaat-adhesines bevat (Hoofdstuk 3).

In een eerste studie die beschreven werd in Hoofdstuk 1, werd de efficaciteit bepaald van twee geïnactiveerde vaccins die de Apx toxines van *A. pleuropneumoniae* bevatten (Hemopig™ Biokema, Lausanne, Zwitserland en Porcilis™ App, Intervet, Boxmeer, Nederland). Hiertoe werden 10 varkens tweemaal, met een interval van 3 weken ingespoten met Hemopig™ en 8 varkens werden tweemaal, met een interval van 3 weken gevaccineerd met Porcilis™ App. Tien andere biggen werden met een gebufferde zoutoplossing op analoge wijze geïnoculeerd en dienden als controles. Drie weken na de tweede vaccinatie werden alle dieren endobronchiaal geïnoculeerd met 10^5 kolonie-vormende eenheden (kve) van een *A. pleuropneumoniae* serotype 9 stam. Alle dieren werden klinisch gevolgd. Dieren die overleefden, werden 7 dagen na de inoculatie geëuthanaseerd. Een verhoogde ademhalingsfrequentie en/of koorts kon worden waargenomen bij alle gevaccineerde dieren en controledieren. Alle controledieren overleefden de besmetting. Zowel in de Hemopig™ groep als de Porcilis™ App groep stierf er 1 dier na de besmetting. Het gemiddeld percentage aangetast longweefsel bedroeg 34% in de controlegroep, 16% in de Hemopig™ groep en 17% in de Porcilis™ App groep. *A. pleuropneumoniae* kon uit de longen van alle controledieren geïsoleerd worden en de gemiddelde bacteriële titer in de caudale longkwabben bedroeg 1.4×10^6 kve/gram. Bij de Hemopig™ groep kon *A. pleuropneumoniae* bij 7 van de 10 dieren geïsoleerd worden en de gemiddelde bacteriële titer bedroeg 1.7×10^3

kve/gram. Bij de Porcilis™ App groep kon *A. pleuropneumoniae* bij 5 van de 8 dieren geïsoleerd worden en de gemiddelde bacteriële titer bedroeg 4.8×10^3 kve/gram. In beide vaccingroepen was het gemiddeld aantal dagen met dyspnee, het gemiddeld aantal dagen met koorts, het gemiddeld percentage aangetast longweefsel en de gemiddelde bacteriële titer in de caudale longkwabben significant lager dan bij de controlegroep. Er werden geen significante verschillen gevonden tussen de 2 vaccingroepen. Er werd geconcludeerd dat beide vaccins partiële bescherming induceerden.

In een tweede studie werd de doeltreffendheid bepaald van een vaccin dat niet alleen Apx toxines maar ook transferrine bindende proteïnes bevat. Hiertoe werden 10 dieren tweemaal gevaccineerd met het vaccin met een interval van 4 weken. Acht controledieren werden tweemaal ingespoten met een gebufferde zoutoplossing. Drie weken na de tweede vaccinatie werden alle dieren endobronchiaal geïnoculeerd met $10^{6.5}$ kolonie-vormende eenheden (kve) van een *A. pleuropneumoniae* serotype 9 stam. In de vaccingroep stierf geen enkel dier. Slechts 1 dier overleefde de besmetting in de controlegroep. De dieren die de besmetting overleefden, werden na 7 dagen geëuthanaseerd. Het gemiddeld percentage aangetast longweefsel bedroeg 64% in de controlegroep en 17% in de vaccingroep. *A. pleuropneumoniae* kon uit de longen van alle dieren geïsoleerd worden. De gemiddelde bacteriële titers in de caudale longkwabben bedroeg 5.0×10^8 kve/gram bij de controlegroep en 3.0×10^6 kve/gram bij de vaccingroep. Er werd geconcludeerd dat het vaccin partiële bescherming induceerde tegen een ernstige besmetting.

Aangezien de pathogenese van pleuropneumonie zeer complex is, kan het insluiten van andere bacteriële virulentiefactoren in subunit vaccins waardevol zijn. Hierbij kan zeker gedacht worden aan adhesines. Het is immers zo dat *A. pleuropneumoniae* zich vasthecht aan de alveolaire epitheelcellen vooraleer er longletsels ontstaan. Aanwezigheid van antistoffen die deze adhesie verhinderen, zouden de eerste stap in de pathogenese kunnen blokkeren en het dier beschermen tegen longinfectie. Om deze reden werd de adhesie van *A. pleuropneumoniae* aan alveolaire epitheelcellen *in vitro* en *in vivo* bestudeerd (Hoofdstuk 2).

In een eerste studie werd de adhesie van *A. pleuropneumoniae* biovar 1 serotype 2, 5a, 9 en 10 aan *in vitro* culturen van alveolaire epitheelcellen onderzocht. Voor de serotypes 5a, 9 en 10 stammen kon een optimale adhesie slechts bekomen worden wanneer de kiemen werden opgegroeid op een NAD-arm milieu (0.001% NAD). Deze groeiomstandigheden waren ook gerelateerd met de expressie van een 55 kDa buitenste membraan eiwit en fimbriae. Voor de serotype 2 stam waren adhesie en expressie van de fimbriae en het 55 kDa buitenste membraan eiwit minder afhankelijk van de groeiomstandigheden. De N-terminale aminozuur sequentie van het 55 kDa buitenste membraan eiwit werd bepaald en er werd geen homologie gevonden met andere gekende sequenties. Dit duidt erop dat het hier gaat om een tot nu toe ongekend eiwit. De adhesie capaciteit van de kiemen was significant lager na

behandeling van de bacteriën met proteolytische enzymen of hitte. Deze bevindingen suggereren dat eiwitten betrokken zijn in de adhesie. Behandeling met tetramethylureum, een product dat hydrofobe verbindingen verbreekt, kon de adhesie niet inhiberen. Behandeling van de bacteriën met natriummetaperiodaat resulteerde in lagere adhesiescores voor de serotype 2 en 9 stammen maar de inhibitie was duidelijk lager dan de inhibitie bekomen na behandeling met proteolytische enzymen. Dit duidt erop dat, naast eiwitten, ook suikers een rol spelen in de adhesie van *A. pleuropneumoniae* aan alveolaire epitheelcellen. Het feit dat behandeling van de bacteriën met een combinatie van natriummetaperiodaat en pronase resulteerde in de hoogste inhibitie suggereert eveneens dat er meer dan één adhesine betrokken is.

In een tweede studie werd het effect bestudeerd van de cultuurcondities op adhesie van geïnactiveerde *A. pleuropneumoniae* biotype 1-serotype 2 en 9 stammen aan de alveolen van experimenteel besmette biggen. Er kon geen verschil in adhesie gevonden worden tussen bacteriën die opgegroeid waren op een NAD-arm of een NAD-rijk medium. Deze groeicondities hadden ook geen effect op de *in vitro* associatie van de bacteriën met surfactant eiwitten. Het is mogelijk dat verschillende adhesines betrokken zijn in de kolonisatie van de alveolen bij geïnfecteerde varkens. In een eerste stap zouden de kiemen zich kunnen associëren met surfactant. Daarna zou een meer stabiele adhesie aan alveolaire epitheelcellen kunnen optreden.

Tenslotte werd de doeltreffendheid van twee bacterins die een *A. pleuropneumoniae* serotype 10 stam bevatten, nagegaan (Hoofdstuk 3). Bacterin 1 bestond uit bacteriën die opgegroeid waren in een NAD-rijk milieu (lage adhesie capaciteit aan alveolaire epitheelcelculturen), bacterin 2 uit bacteriën die opgegroeid waren in een NAD-arm milieu (hoge adhesie capaciteit aan alveolaire epitheelcelculturen). Tien dieren werden tweemaal gevaccineerd met het bacterin 1 en 9 dieren tweemaal met het bacterin 2, telkens met een interval van 3 weken. Tien controledieren werden tweemaal ingespoten met gebufferde zoutoplossing. Drie weken na de tweede vaccinatie werden alle dieren endobronchiaal geïnoculeerd met $10^{6.5}$ kolonievormende eenheden (kve) van een *A. pleuropneumoniae* serotype 10 stam. Drie dieren van de bacterin 1 groep en 2 dieren van de bacterin 2 groep stierven na de besmetting. Slechts 2 van de controledieren overleefden de besmetting. De dieren die de besmetting overleefden, werden na 7 dagen geëuthanaseerd. Het percentage dieren met erge longletsels ($>10\%$ van de long aangetast) bedroeg 100% bij de controlegroep, 70% bij de bacterin 1 groep en 22% bij de bacterin 2 groep. *A. pleuropneumoniae* kon uit de longen van alle dieren geïsoleerd worden. De gemiddelde bacteriële titer in de caudale longkwabben bedroeg 7.0×10^6 kve/gram bij de controlegroep, 6.3×10^5 kve/gram bij de bacterin 1 groep en 1.3×10^6 kve/gram bij de bacterin 2 groep. Er werd geconcludeerd dat beide bacterins een partiële bescherming bieden tegen een ernstige besmetting. Bovendien waren er indicaties dat het bacterin 2, dat

A. pleuropneumoniae kiemen bevat opgegroeid onder condities die resulteerden in hoge *in vitro* adhesie, een betere bescherming geeft dan het bacterin 1.

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“Anyone who has never made a mistake has never tried anything new”

-Albert Einstein-

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If not for you,
Babe, I couldn't find the door,
Couldn't even see the floor,
I'd be sad and blue,
If not for you.

If not for you,
Babe, I'd lay awake all night,
Wait for the mornin' light
To shine in through,
But it would not be new,
If not for you.

If not for you
My sky would fall,
Rain would gather too.
Without your love I'd be nowhere at all,
I'd be lost if not for you,
And you know it's true.

If not for you
My sky would fall,
Rain would gather too.
Without your love I'd be nowhere at all,
Oh! What would I do
If not for you.

If not for you,
Winter would have no spring,
Couldn't hear the robin sing,
I just wouldn't have a clue,
Anyway it wouldn't ring true,
If not for you.

-Bob Dylan-

CURRICULUM VITAE

Ingrid Van Overbeke werd op 1 februari 1971 geboren te Gent. In 1989 beeïndigde zij haar humaniora-opleiding aan het Maria Middelaars Instituut te Aalter, richting wiskunde-latijn, en werd de studie Diergeneeskunde aangevat aan de Universiteit Gent. In 1993 werd het diploma van “Kandidaat in de Diergeneeskundige Wetenschappen” behaald. Vervolgens werd in 1996 het diploma “Dierenarts” behaald met onderscheiding.

Na haar studies trad zij in dienst bij de vakgroep Pathologie, Bacteriologie en Pluimveeziekten als wetenschappelijk medewerker in het kader van een IWONL project (Ministerie van Landbouw). Dit onderzoek omvatte studies naar de karakterisatie van de adhesines van *Actinobacillus pleuropneumoniae* en hun rol bij de bescherming van varkens tegenover pleuropneumonie. Deze studies werden uitgevoerd onder leiding van Prof. Dr. F. Haesebrouck en Prof. Dr. R. Ducatelle.

Van oktober 1998 tot juli 2000 volgde zij de specialisatieopleiding proefdierkunde en behaalde het diploma van “Gediplomeerde in de gespecialiseerde studies proefdierkunde” met grote onderscheiding. Tevens volgde zij de doctoraatsopleiding in de diergeneeskundige wetenschappen waarvan het getuigschrift in 2001 bekomen werd.

Sinds 2003 werkt zij aan een onderzoeksmandaat van het Vlaams Instituut voor de bevordering van het Wetenschappelijk-Technologisch Onderzoek in de Industrie.

Ingrid Van Overbeke is auteur of mede-auteur van meerdere publicaties in internationale en nationale tijdschriften en zij was spreker op meerdere internationale congressen.

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