Université de Montréal

STUDY OF THE ADHERENCE OF *ACTINOBACILLUS PLEUROPNEUMONIAE* TO
IMMORTALIZED PORCINE EPITHELIAL CELLS

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Universtié de Montréal

Faculté des études supérieures

Ce mémoire intitulé

STUDY OF THE ADHERENCE OF *ACTINOBACILLUS PLEUROPNEUMONIAE* TO IMMORTALIZED PORCINE EPITHELIAL CELLS

Présenté par

ELIANE AUGER

À été évalué par un jury compose des personnes suivantes

Michaël Mourez, président-rapporteur

Mario Jacques, directeur de recherche

Marcelo Gottschalk, membre du jury

RÉSUMÉ

Nous avons standardisé un modèle *in vitro* d'adhérence pour l'étude des pathogènes respiratoires du porc en utilisant deux lignées cellulaires porcines nouvellement établies, notamment la lignée de cellules de trachée Newborn Pig Trachea (NPTr) et la lignée de cellules de poumon St. Jude Porcine Lung (SJPL). Avec l'aide de ce modèle, nous avons étudié les propriétés d'adhérence et d'invasion de l'agent étiologique de la pleuropneumonie porcine, *Actinobacillus pleuropneumoniae*, ainsi que d'autres *Pasteurellaceae* du porc comme *Haemophilus parasuis*, *Actinobacillus suis* et *Pasteurella multocida*. Nous avons observé des différences d'adhérence entre les souches ainsi qu'entre les lignées. La croissance d' *A. pleuropneumoniae* serotype 1 sous conditions restreinte en fer ou en NAD n'a pas affecté son adhérence aux deux lignées. Au niveau de l'invasion, la souche d' *A. pleuropneumoniae* testée n'envahie pas les cellules dans notre modèle tandis que les autres *Pasteurellaceae* ont la capacité d'envahir les cellules. La production de cytokines pro-inflammatoires par les deux lignées suite à une induction par des bactéries *A. pleuropneumoniae* inactivées a démontré une augmentation de la production de IL-8 par les cellules NPTr, mais aucune production d'autres cytokines n'a été détectée pour les deux lignées. Une analyse des cellules SJPL en contact avec *A. pleuropneumoniae* suggère une surexpression de protéines impliquées dans l'apoptose et une répression de protéines impliquées dans la croissance cellulaire. Ces résultats démontrent le grand potentiel de ces lignées pour l'étude de la pathogenèse des pathogènes respiratoires du porc.

MOTS-CLÉ : porc, adhérence, invasion, cytokines, lignée cellulaire, poumons, trachée.

ABSTRACT

We have developed an *in vitro* adherence model for the study of respiratory pathogens of swine using two newly established porcine respiratory tract cell lines, namely the Newborn Pig Trachea (NPTr) and the St. Jude Porcine Lung (SJPL) cell lines. Using this model, we studied the adherence and invasion abilities of the etiological agent of porcine pleuropneumonia, *Actinobacillus pleuropneumoniae*, as well as other porcine *Pasteurellaceae* such as *Actinobacillus suis*, *Heamophilus parasuis*, and *Pasteurella multocida*. We observed differences in adherence between strains and between cell lines. Growth of *A. pleuropneumoniae* serotype 1 under iron- or NAD-deprived conditions did not affect the adherence to both cell lines. The strain of *A. pleuropneumoniae* tested did not invade porcine epithelial cells in our *in vitro* model while the other porcine *Pasteurellaceae* have the capacity to invade. The production of pro-inflammatory cytokines by both cell lines following an induction with killed *A. pleuropneumoniae* revealed an increased production of IL-8 by the NPTr cells, while no detectable levels were noticed for the other cytokines in either cell lines. Protein profiling of the SJPL cells revealed a tendency for the up-regulation of apoptosis proteins and for the down-regulation of cell growth proteins. Altogether, these results demonstrate the great potential and versatility of these cell lines in the study of the pathogenesis of porcine respiratory tract pathogens.

KEY WORDS: swine, adherence, invasion, cytokines, cell line, lung, trachea.

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LIST OF ABBREVIATIONS

A. actinomycetemcomitans	*Actinobacillus actinomycetemcomitans*
A. pleuropneumoniae	*Actinobacillus pleuropneumoniae*
APX	*A. pleuropneumoniae* RTX
A. suis	*Actinobacillus suis*
BHI	Brain Heart Infusion
DMEM	Dubelcco's Modified Eagle Medium
DPBS	Dublecco's Phosphate Buffer Saline
E.coli	*Escherichia coli*
EDDHA	Ethylenediamine dihydroxyphenyl acetic acid
ELISA	Enzyme linked immunoabsorbent assay
EPS	Exopolysaccharide
FBS	Fetal Bovine Serum
fhu	Ferric hydrosamate uptake
H. influenzae	*Haemophilus influenzae*
H. parasuis	*Haemophilus parasuis*
HgbA	Hemoglobin binding protein A
IVET	In vivo expression technology
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
MEM	Minimum Essential Medium
NAD	Nicotinamide adenine dinucleotide
NPTr	Newborn Pig Trachea
OMP	Outer membrane protein

ORF	Open reading frame
PGA	Poly-beta-1,6-N-acetyl-D-glucosamine
PIA	Polymer of beta-1,6-linked N-acetylglucosamine
P. multocida	*Pasteurella multocida*
PPLO	Pleuropneumonia-like organisms medium
RTX	Repeats in the structural toxin
SCOTS	Selective capture of transcribed sequences
SJPL	St. Jude Porcine Lung
STM	Signature-tagged mutagenesis

To my parents

Lyne Guillot & Marc Auger

For believing in me

and

For providing me with all the tools to succeed

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These last two years will definitely stay engraved in my memory for the rest of my life, and that is due to all the people that surrounded me and supported me in this journey. I owe these two years of realisations, achievement and growth to Dr. Mario Jacques, my research director, for giving me the opportunity to live this experience in his laboratory amongst a great group of people I'm now proud to call my friends. He also provided me with great insight and knowledge of the research world.

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I. INTRODUCTION

Porcine respiratory diseases have heavily impacted the economy of the pig rearing industry worldwide (103). *Actinobacillus pleuropneumoniae*, exemplar of these porcine respiratory pathogens, causes porcine pleuropneumonia by colonising the respiratory tract of swine (103). The colonisation of the host is known to be the first step of infection and is initiated by the adhesion of the bacteria to the host cells (92).

For this reason, numerous models have been used to study the pathogenesis of this porcine pathogen, like *in vivo* models, primary cell models including tracheal, bucal and lung epithelial cells as well as tissue section including frozen tracheal and lung cross-sections (15, 45, 51, 109). These models and other experiments led to the discovery of LPS as the only known adhesin of *A. pleuropneumoniae* as well as the observation of many putative adhesins (7, 11, 31, 48, 51, 62, 82, 90, 109, 114). To the best of our knowledge, however, no models using immortalised cells lines, which are known to be very effective and advantageous in other models, have been used to study *A. pleuropneumoniae*.

The goal of this present study is therefore: (1) to standardise immortalised cell line infection model to study the adherence and the invasive capacities of *A. pleuropneumoniae* and other porcine *Pasteurellaceae* using two newly established porcine respiratory tract cell lines; namely the Newborn Pig Trachea (NPTr) (33) and the St. Jude Porcine Lung (SJPL) (94) cell lines; and (2) to study the response of the cells to their interaction with *A. pleuropneumoniae* by calculating pro-inflammatory cytokine production and by profiling protein expression using antibody microarray technology.

II. LITERATURE REVIEW

Actinobacillus pleuropneumoniae

1. History

Formerly known as *Haemophilus pleuropneumoniae* or *Haemophilus parahaemolyticus*, *Actinobacillus pleuropneumoniae* is a member of the *Pasteurellaceae* family and the etiologic agent of porcine pleuropneumonia (103). The disease was first identified in the United States in 1957 and then made its way around the world, helped by the industrialization of swine production (25, 83). It is now present in most pig-keeping countries including many European countries, the United States, Mexico, South America, Canada, Japan, Korea, Taiwan and Australia (103, 112).

2. Characteristics

A. pleuropneumoniae is a small Gram-negative capsulated rod with coccobacillary morphology and is highly specific to its host, the swine (43, 112). A high dose however, has been shown to be able to infect rats and mice (45). *A. pleuropneumoniae* is a non-motile facultative anaerobe that does not produce spores (13). Biochemically, it is a beta-hemolytic pathogen (103). In addition, it can degrade urea, reduce nitrates and nitrites, acidify fructose, glucose, maltose, manitol, xylose and mannose, but cannot produce gas during glucose fermentation and inositol and inuline acidification (13).

3. Classification

The two biotypes of *A. pleuropneumoniae* are determined by their NAD requirement; biotype 1 regrouping NAD-dependent serotypes, and biotype 2 regrouping the serotypes capable of synthesising NAD from specific pyridine nucleotides or their precursor (18). Surface polysaccharide antigens determine the different serotypes of each biotype. Biotype 1 includes serotypes 1 to 12 and 15, while biotype 2 includes serotypes 2, 4, 7, 9, 13 and 14. It is important to note that two biotype 1 strains belonging to the serotype 13 have been isolated recently. Serotypes 5 and 1 are subdivided in serotype 1a, 1b, 5a and 5b based on minor differences in the polysaccharides. The serotypes 1, 5, 9, 10, and 11 seem to be more virulent than the other serotypes (103). The most prominent serotypes in North America are serotypes 1, 5 and 7. In Europe, the primary serotype found is serotype 2 while serotype 15 predominates in Australia (14, 112).

4. Etiology of porcine pleuropneumonia

Porcine pleuropneumonia is a very contagious and often fatal disease. *A. pleuropneumoniae* colonises the respiratory tract of pigs to cause this disease, characterised by necrotic and hemoragic lung lesions, coughing, severe respiratory distress and more (18). Four stages of the disease have been identified; peracute, acute, subacute and chronic (103). During infection, cytokines, including IL-1β, IL-8 and TNF-α, are detected in alveolar fluid and tissue lesions (103).

In the peracute phase, sudden onset of the disease is observed in one or more pigs in the same or different pens. Although fever is present (41.5°C), no major

respiratory signs are noticed. Slight diarrhea and vomiting as well as apathy and anorexia are the major symptoms of this phase of the disease (103).

In the acute phase, a high morbidity and mortality are often observed as well as severe cardiac and respiratory distress. Many pigs in the same or different pens are usually affected (103). Lung lesions are characteristic of the acute and preacute phase and lead to oedema, inflammation, hemorrhage and necrosis (18).

In the subacute phase as well as in the chronic form all the acute signs have disappeared and the pigs are left with only spontaneous or intermittent cough (103). Weight loss due to loss of appetite is also a characteristic feature of chronic cases (112).

After the infection has established in a herd, continuous apparition of these symptoms occurs as well as, periodically, a reappearance of acute cases (25).

5. Treatments and Prevention

The use of penicillin and other penicillin analogs are usually effective against *A. pleuropneumoniae* infections, even though plasmid-mediated antibiotic resistance is being identified more frequently, especially in serotypes 1, 3, 5, and 7 (32, 103). Resistance to ampicillin, streptomycin, sulfonamides, tetracyclines and chloramphenicol is of great concern. Indeed, low MIC values have been observed *in vitro* for penicillin, ampicillin, cephalosporin, chloramphenicol, tetracylcines, colistin, sulphonamide, cotrimoxazole and gentamicine while high MIC values were observed for streptomycin, kanamycin, spectinomycin, spiramycin and lincomycin. The major problem in using antibiotic treatments is the pattern of herd infection.

Antibiotics are only effective in the initial phase of the disease and since the animals are anorexic during infection, the antibiotic must be given by injection and should be given repeatedly to insure high concentrations in the blood (103). Mass treatment is therefore needed and the rapid course of the disease renders the treatment a matter of timing and of limited value (32). In addition, although clinical signs may cease, chronic infections in lung abscesses or on tonsils of carriers persist after treatment and are an important source of infection for other animals (103).

Many vaccine formulations have been produced with the goal of generating efficient protection against *A. pleuropneumoniae* infections. Whole-cell bacterins reduced mortality, but do not protect against heterologous serotypes. Sub-unit vaccines seem to confer a better cross-protection than bacterins, decreasing clinical symptoms and increasing performance of the animals. Apx toxins have been shown to be an important part of sub-unit vaccine but are not sufficient on their own. Other factors are evidently involved in protective immunity (44). Extra-cellular products, iron-binding proteins, and outer membrane proteins are other antigens which have been found to induce protection from *A. pleuropneumoniae* infections (103). Adhesins would be great vaccine candidates as they are immunogenic and since adhesion is the first step in the pathogenesis of porcine pleuropneumonia. The predicament however, is that the virulence factors responsible for adhesion of *A. pleuropneumoniae* are poorly known (103). LPS, the only known adhesin of *A. pleuropneumoniae*, have been shown to induce protection (89).

Prevention is without a doubt the key solution to avoid outbreaks and consequently avoiding death losses as well as monetary losses. Thorough cleaning of barns and rooms as well as an all-in and all-out management of animal is of great importance and has contributed to a decrease in infection of younger animals by virulent strains of older animals (32). New animals should be derived or originate from a disease-free herd and should be held in quarantine prior to introduction to the new herd (103). Canadian workers implemented a program in which animals of common immune status are commingled so that populations are compatible in an immunological point of view. Three categories have been formed; (1) serologically positive for *A. pleuropneumoniae* without a history of clinical disease, (2) serologically negative and clinically free of *A. pleuropneumoniae* and (3) history of clinical disease caused by *A. pleuropneumoniae* which has been pathologically and microbiologically confirmed. This program greatly decreased the risk of porcine pleuropneumonia and showed growth performance improvement as well as a decrease in disease outbreaks (32).

6. Virulence Factors of *A. pleuropneumoniae*

The virulence of *A. pleuropneumoniae* is accomplished by the help of many factors including exotoxins, endotoxin, capsule, outer membrane proteins, adhesins, transferrin binding proteins and other iron-acquisition systems. These virulence factors which contribute to the different stages of *A. pleuropneumoniae* infection are discussed in detail below.

6.1 Exotoxins

A. pleuropneumoniae produces four different type of toxins which are all part of the RTX (repeat in the structural toxin) family of toxins and are consequently called Apx (*A. pleuropneumoniae* RTX toxin). RTX toxins are pore-forming proteins that are greatly cytolytic and common in Gram-negative pathogens (35).

ApxI is the most hemolytic and cytolytic of all Apx and therefore greatly cytotoxic for phagocytic cells (35). It was formerly named cytolysin I or hemolysin I (43). The *apxI* operon contains four genes place in the order *apxICABD* (35). *ApxIC* being the activator gene, *apxIA* the pretoxin structural gene, and *apxIB* and *apxID* the secretion apparatus genes (35). The transcriptional activity of the *apxI* operon can be greatly enhanced by the addition of calcium in the growth medium (36). The ApxI is a 105 kDa protein and is expressed by serotypes 1, 5a, 5b, 9, 10 and 11 (35).

ApxII is weakly hemolytic and cytotoxic and is a also 105 kDa protein (35). The ApxII toxin was formerly known as cytolysin II or hemolysin II (43). It is expressed by all serotypes except serotypes 10 and 14, and is encoded by the *apxII* operon which has the activator gene and the structural gene but lacks the secretion apparatus genes. ApxII seems to use the products of the *apxIBD* genes for export (35).

Encoded by the *apxIII* operon, ApxIII is a 120 kDa protein which is strongly cytolytic but not hemolytic (60). None of the biotype 2 strains express this toxin and only serotypes 2-4, 6 and 8 of biotype 1 express it (35). It was formerly known as pleurotoxin (43).

ApxIV is weakly hemolytic and has a co-hemolytic (CAMP) effect similarly to the effects of ApxII but is only expressed *in vivo*. Other than the unusual C-terminal end, ApxIVA has all the structural characteristics of RTX toxins (93).

Table 1. Distribution of the APX toxins genes in the different serotypes of *A. pleuropneumoniae* (35, 102).

Serotypes	Secreted toxins	Genes			
Biotype 1		*apxI*	*apxII*	*apxIII*	*apxIV*
1, 5, 9, 11	ApxI, ApxII, ApxIV	*CABD*	*CA*	None	*A*
10	ApxI, ApxIV	*CABD*	None	None	*A*
2, 4, 6, 8, 15	ApxII, ApxIII, ApxIV	*BD*	*CA*	*CABD*	*A*
3	ApxIII, ApxIV	None	*CA*	*CABD*	*A*
7, 12	ApxII, ApxIV	*BD*	*CA*	None	*A*
Biotype 2		*apxI*	*apxII*	*apxIII*	*apxIV*
2, 4, 7, 13	ApxII, ApxIV	*BD*	*CA*	None	*A*
9	ApxI, ApxII, ApxIV	*CABD*	*CA*	None	*A*
14	ApxI, ApxIV	*CABD*	None	None	*A*

The toxins secreted *in vivo* as well as the presence of the different *apx* genes in the different serotypes of *A. pleuropneumoniae* are portrayed in Table 1.

ApxI and ApxII have both shown to be maximally expressed when cell density increases and when growth rate decreases, indicating that a mechanism could be used by the bacteria to prevent high-level expression of these toxins until an infection is established (57). It was shown, in *A. pleuropneumoniae* serotype 1, that Apx toxins are released from the cells into the culture medium by small vesicles of about 20 to 200 nm. The authors also mention the presence of many proteases in these vesicles and that other serotypes of *A. pleuropneumoniae* may use the same system to release their Apx toxins (77).

The pore forming activities of ApxI, ApxII and ApxIII were studied and revealed similarities between ApxI and ApxIII in that they both created the same amount of pores in aqueous-phase-bathing lipid bilayer membranes, but the pores of ApxIII were smaller than that of ApxI; 1.8 nm as compared to 2.4 nm for ApxI. ApxII were able to produce pores of similar size to those of ApxI (2.5 nm) but with a 10 times weaker formation ability (70).

The virulence of the different serotypes coincides greatly with the presence of the Apx toxins, especially ApxI and ApxII. In fact, serotypes 1, 5, 9 and 11 are known to be especially virulent and all express both ApxI and ApxII. Apx toxins are a major virulence factor as they hinder the host's immune defences and render the strain virulent (35). They are essential in pathogenesis of porcine pleuropneumonia (43).

6.2 Capsule

A. pleuropneumoniae is an encapsulated pathogen. The capsule, responsible for the iridescence of the colonies on clear agar medium, is present on all strains and is between 18 to 230 nm thick depending of the serotype (30, 43). Serotypes 5a, 5b and 10 consist of repeating oligosaccharide units while serotypes 2, 3, 6, 7, 8, 9 and 11 consist of teichoic acid polymers joined by phosphate diester bounds and serotypes 1, 4 and 12 consist of oligosaccharide polymers joined through phosphate bounds. The structure of the serotype 1 capsular polysaccharide is illustrated in Figure 1.

$$\left[-4)\text{-}\beta\text{-}D\text{-}\underset{\substack{6\\\uparrow\\1\\(Ac)_{0.85}}}{G}lcNAcp\text{-}(1-6)\text{-}\alpha\text{-}D\text{-}Galp\text{-}(1-O-\overset{\overset{O}{\|}}{\underset{\underset{OH}{|}}{P}}-O- \right]_n$$
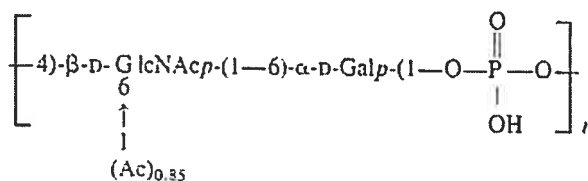
Figure 1. The capsular polysaccharide from *A. pleuropneumoniae* serotype 1. Adapted from Altman *et al.*, 1985.

The genetic locus responsible for capsular polysaccharide export contains four genes arranged in the order *cpxDCBA*, while the synthesis genes are arranged in the order *cpsABCD* (30). The capsule is not as major an immunologic factor as the Apx toxins but is certainly required for virulence (43). In fact, a correlation has been observed between the thickness of the capsule and the virulence of the strain (30). Indeed, capsule-deficient mutants of serotypes 1 and 5 were less pathogenic than the

parental strains. An acapsular mutant of serotype 1 also showed increased adherence to tracheal cross sections indicating that the capsule does not have a role in adhesion (91). Essentially, this information reveals that although the capsule may mask certain adhesins, rendering the bacteria less adherent, the capsule protects the bacteria greatly from host defences (30, 91).

6.3 Endotoxin: Lipopolysaccharide

This surface polysaccharide is a complex molecule composed of three distinct regions; (i) lipid A, (ii) core-oligosaccharide and (iii) O-antigen (Figure 2).
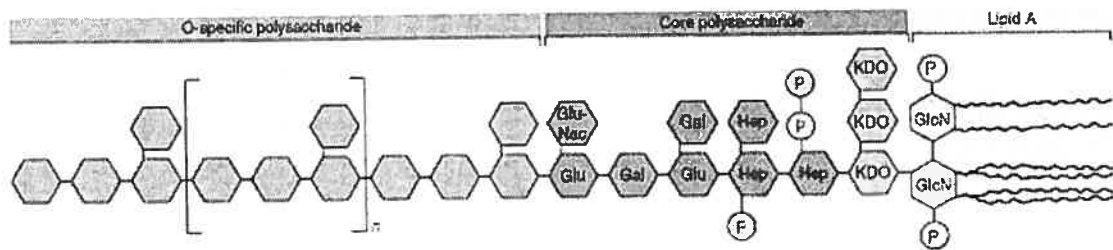
Figure 2. Structure of lipopolysaccharides. Adapted from Brook *et al.* (2003).

The O-antigen, the most variable part of the LPS, is responsible for the characteristic smooth, semi-rough or rough phenotype of the different serotypes of *A. pleuropneumoniae* which depends on the amount of repeated polysaccharide chains present. Serotypes 2, 4 and 7 are smooth, serotype 1 and 5 are semi-rough, while serotypes 3 and 6 are rough (11, 21). It is the core oligosaccharide region, however, which seems to be necessary for optimal adherence (see section 6.5.1) and is composed of 3-deoxy-D-mano-2-octulosonic acid (49). It is linked covalently to the

lipid A and is made up of an outer and an inner core (48). Two types of core oligosaccharide have been suggested; type I and II (55, 73). Based on electrophoresis mobility, serotypes 1, 6, 9, and 11 have a core of type I while the other serotypes have a core of type II (55). The structure of this region is represented in Figure 3 below.

R'   α-Glc II-(
| |
▼   ▼
6   6
R→4)-D-α-D-Hep IV-(1→6)-β-Glc I-(1→4)-L-α-D-Hep I-(1→5)-α-Kdo
                                          3
                                          ▲
                                          |
            L-α-D-Hep III-(1→2)-L-α-D-Hep II-(1
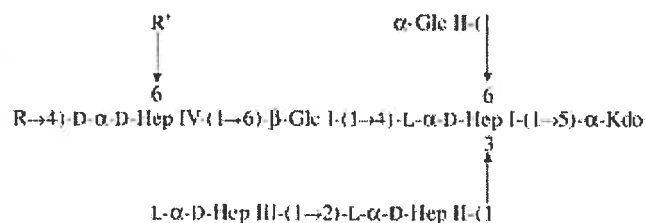
Figure 3. Structure of the core oligosaccharide region of *A. pleuropneumoniae*. The R and R' region differ in different serotypes. For serotype 1, R is (1*S*)-Gal*a*NAc-(1→ 4,6)-α-Gal II-(1→ 3)-β-Gal I-(1→, and R' is H. Adapted from St. Michael *et al.* (2004).

The serotype 1 O-antigen is composed of branched tetrasaccharide repeating units composed of 4 residues; 2 α-L-rhamnopyranosyls, 1 α-D-glycopyranosyl and 1 2-acetamido-2-deoxy-β-D-glucose (3). The structure is illustrated in Figure 4. The O-antigen does not however appear to play a role in virulence but seems to be implicated in serum resistance and in binding to phosphatidylethanolamine (58, 63, 90). Interestingly, a field isolate of *A. pleuropneumoniae* serotype 1 with a truncated outer core and no O-antigen was still virulent in pigs (53).

$$\left[6)\text{-}\alpha\text{-}\text{D-Glc}p\text{-}(1\!-\!2)\text{-}\alpha\text{-}\text{L-Rha}p\text{-}(1\!-\!2)\text{-}\alpha\text{-}\text{L-Rha}p\text{-}(1\right]_{n}$$

$$\begin{array}{c} 3 \\ \uparrow \\ 1 \\ \beta\text{-}\text{D-GlcNAc}p \end{array}$$
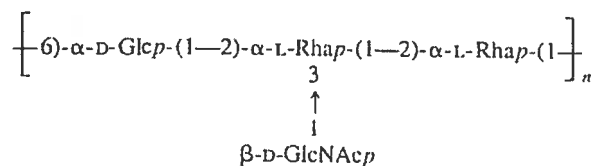
Figure 4. Structure of the O-antigen of *A. pleuropneumoniae* serotype 1. Adapted from Altman et al. (1986).

The lipid A consists of about 9.2% of the total lipopolysaccharide and is predominantly formed of C14:0, C16:0 and 3-hydroxy C14:0 fatty acids (30). The *A. pleuropneumoniae* lipid A, as well as its fatty acid components, are able to bind porcine hemoglobin yet the significance of this molecule in an *in vivo* iron acquisition system is not clear (18). Liposomes containing lipid A were shown to provide protection from death and severe lesions adding support to the role of the lipid A in the pathogenesis of *A. pleuropneumoniae* (30).

Purified LPS are known to be able to cause damage to cells and tissues. However, *A. pleuropneumoniae* LPS are not the cause of the typical lesions seen in porcine pleuropneumonia, although they may contribute to their formation by increasing the effects of the Apx toxins (43).

6.4 Biofilm

A. pleuropneumoniae has first been reported to create biofilm in 2004 by Kaplan *et al.*(62). Biofilms are structured community of bacterial cells enclosed in a self-produced polymeric matrix. They are formed by initial attachment of bacterial cells to a solid surface, followed by the arrangement of micro-colonies which then differentiate into exopolysaccharide-encased mature biofilms (22). This exopolysaccharide (EPS) encasement traps nutriments, helps prevent detachment and protects the bacteria from biocides and from the immune system of the host (68). One of the best studied EPS is a hexosamine-containing polymer called PIA in *Staphylococcus* species and PGA in *E. coli.* (111). *Actinobacillus actinomycetemcomitans* and *A. pleuropneumoniae* both produce PGA, a linear polymer of N-acetyl-D-glucosamine residues in β (1, 6) linkage (62).

Following the observation of biofilms in *A. pleuropneumoniae*, Kaplan *et al.* studied the prevalence of this phenotype in other strains. Out of the 15 reference strains tested only the L20 reference strain of serotype 5b and the 56513 reference strain of serotype 11 exhibited biofilm formation but about half of all field isolates of serotypes 1, 5 and 7 where observed to produce biofilms. It was also observed that after one or two passages in fresh broth, the phenotype became irreversibly lost (61).

6.5 Adhesins

The *A. pleuropneumoniae* adhesion mechanism is still poorly known. LPS are the only known adhesins of *A. pleuropneumoniae*, all others being putative adhesins. Below, the adhesin and putative adhesins of *A. pleuropneumoniae* are described in detail.

6.5.1 Lipopolysaccharide (LPS)

Folowing the observation that *A. pleuropneumoniae* is able to adhere to frozen lung sections as well as porcine tracheal sections, Jacques *et al.* was able to show that the LPS are the major molecule responsible for this adhesion phenomenon (11, 12, 48, 51, 82). Isogenic mutants of the serotype 1 reference strain 4074 have also been created by Jacques *et al.* using mini-Tn10 transposons. These mutants were then characterised to investigate which region of the LPS is responsible for the adhesion. The core region, especially the galactose and D,D-heptose residues of the outer core, was shown to be essential for adherence (39, 81, 86, 88). A mutation in the core region caused a decreased adherence while a mutation in the O-antigen did not, demonstrating that indeed, the core region and not the O-antigen is responsible for adherence. As for the lipid A region, purified LPS that did not contain the region were still able to block adherence of *A. pleuropneumoniae* to frozen tracheal cross-sections demonstrating that the lipid A is not necessary for adherence (82).

Although a 38.5 kDa putative LPS-binding receptor has been identified for *A. pleuropneumoniae*, no definite receptors have been described to date (82).

6.5.2 Type IV fimbriae

Fimbriae were first noticed on *A. pleuropneumoniae* by Utera *et al.*, but were suspected since 1988 (107). These fimbriae were later shown to be group A type IV fimbriae or pili (114). Type IV fimbriae are present on many Gram-negative bacterial pathogens and can serve many functions like adhesion and twitching motility. In addition, it has been shown that proteins of extremely high similarity with the type IV pilins are essential components of biological processes such as extracellular protein secretion. Type IV fimbriae are characterized according to similarities in amino acid sequence of the pilin polypeptide and the occurrence of N-methylated amino acids in the first amino acid of the mature pilin structural subunit (104). The fimbriae are only expressed by *A. pleuropneumoniae* under specific conditions which indicates that the expression is regulated by specialised regulatory elements (114). In fact, a study by Overbeke *et al.* showed that fimbriae could be detected on serotypes 2, 5a, 9 and 10 when grown under NAD-restricted condition while they were only detected on serotype 2 and not serotype 5a, 9 and 10 when grown under NAD-rich conditions (109). The fimbrial subunit of *A. pleuropneumoniae*, called ApfA is a 17 kDa protein and highly similar to type IV fimbrial subunit of other bacterial pathogens and highly conserved between 12 representative serotypes of *A. pleuropneumoniae* (101, 114). The genes allowing biosynthesis of these fimbriae are arranged in an operon with the order *apfABCD* as shown in Figure 5 (16).
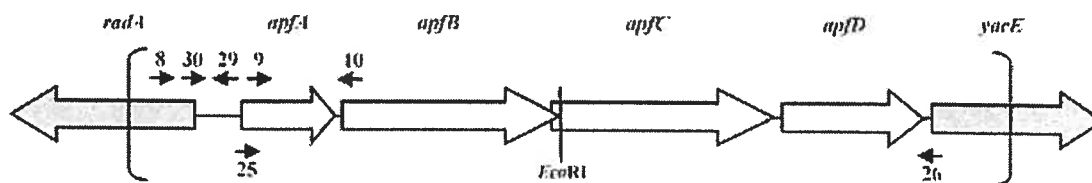
Figure 5. The type IV fimbrial operon of *A. pleuropneumoniae*. White arrows represent genes of the type IV fimbriae. Adapted from *Boekema et al.* (2004).

The operon is under the regulation of a promoter which is controlled by environmental conditions, such as growth phase and nutriment availability. Host cell contact has also been observed to be correlated to strong fimbriae promoter activity (16).

The type IV fimbria is an immunogenic molecule as well as a putative adhesin which is expressed in different serotypes. This makes it a great potential vaccine candidate and potentially a major virulence factor of *A. pleuropneumoniae* (101).

6.5.3 Outer membrane protein of 60 kDa

A putative adhesin of 60 kDa has been discovered by Enriquez-Verdugo *et al.* in 2004 (31). After showing that all *A. pleuropneumoniae* serotypes tested were capable of adhering to collagen, the authors suggested that an outer membrane protein of 60 kDa would be the collagen binding protein responsible for this adhesion. The authors made this observation using overlay assays of outer membrane proteins (OMP) electroblotted onto a nitrocellulose membrane. They were able to

show that a 60 kDa protein was able to recognize four types of pig-lung collagen. Serotypes 1 to 12 including 5a and 5b were tested and only serotypes 6 and 11 did not express this protein. Following this observation, the authors purified the protein and did an inhibition assay using an anti-OMP_{60}. Cell binding was inhibited in a concentration-dependent effect. Using electron microscopy, the putative protein was observed to be located on the bacterial surface. The authors are working on the identification of the gene encoding this 60 kDa protein which will hopefully lead to the creation of OMP_{60} mutants (31).

6.5.4 Outer membrane protein of 55 kDa

In 2002, Overbeke *et al.* observed a 55 kDa band on a SDS-PAGE gel of the sacrosyl-insoluble OMP fraction of *A. pleuropneumoniae* serotypes 2, 5a, 9 and 10 strains when grown in NAD-restricted condition (109). This band was absent or weaker when bacteria were grown in NAD-rich conditions. Since an increased in adhesion was observed when bacteria were grown in NAD-restricted condition, the authors concluded that this OMP of 55 kDa could be involved in adherence. The adhesion scores of serotype 2 did not differ between the NAD-rich and NAD-restricted conditions. Furthermore, the 55-kDa protein was expressed by this serotype under both conditions. The N-terminal amino acid sequence of this protein was obtained for serotypes 5a and 10 showing no homology with other proteins sequences (109). OMP_{55} mutants should be created and tested in an adhesion model to prove its role in adherence.

6.5.5 PGA

PGA is an exopolysaccharide and a major component of biofilm matrix encoded by the *pgaABCD* locus (62). This linear polymer of N-acetyl-D-glucosamine residue in β (1, 6) linkage is well characterised as PIA in *Staphylococcus* species and PGA in *Escherichia coli* (62, 111). As aforementioned (section 5.4), it is a hexosamine-containing polymer that was recently observed in *A. pleuropneumoniae* (62). Numerous studies revealed PIA or PGA as being a polysaccharide intercellular adhesin (111). Kaplan *et al.* suggests that *A. pleuropneumoniae* biofilms produce a hexosamine-containing intercellular adhesin highly related to the *pgaC*-dependent polysaccharide of *A. actinomycetemcomitans* which is structurally and functionally related to the *E. coli* PGA and *Staphylococcus epidermidis* PIA. PGA does not, however, seem to have an important role in adhesion to surfaces (62).

6.6 Iron acquisition systems

Iron, an essential nutriment for *A. pleuropneumoniae* and almost all living organisms, is scarce in the host's environment since it is usually complexed to glycoproteins such as transferrin and lactoferrin (43). Bacteria have evolved many different systems to acquire iron. Below are explanations of the ones used by *A. pleuropneumoniae* as well as a schematic representation of these systems (Figure 6).

6.6.1 Siderophores

Siderophores are iron chelators that can bind iron present at very low concentrations (68). *A. pleuropneumoniae* has been recognised to secrete a yet unkown type of siderophore when grown in iron depleted medium. It is also believed to express receptors for hydroxamate and catechol siderophores secreted by other microorganisms (18, 28). A 77 kDa protein called FhuA has been identified as the receptor for ferric hydroxamate, a hydroxamate siderophore. FhuA has also been shown to act as a ferrichrome receptor which is not regulated by iron (75). This receptor is coded by the *fhu* operon and is homologous to the *fhuACDB* cluster of *E. coli*. The rest of the *fhu* operon is involved in the transport of the iron source across the cytoplasmic membrane. FhuD being the periplasmic protein that translocate ferric hydroxamate from the outer to the inner membrane and FhuB and FhuC the cytoplasmic-membrane-assosiated proteins and part of the ABC transporter that internalises ferric hydroxamate (74).

6.6.2 Transferrin-binding proteins

A. pleuropneumoniae is able to use transferrin, although solely coming from swine (18). This fact has been suspected to play a role in the high host specificity of *A. pleuropneumoniae* (43). TbpA and TbpB are the two transferrin-binding proteins expressed by *A. pleuropneumoniae* in iron-restricted conditions and are co-transcribed with the *exbBD* genes (18). The *tbpA* and *tbpB* genes are located on the same operon and express the highly immunogenic TbpA and TbpB proteins (106). TbpA is a 100 kDa protein which likely forms a transmembrane channel for transport

across the outer membrane, while TbpB is a 60 kDa protein suspected to be a lipoprotein anchored to the outer membrane. Both TbpA and TbpB bind the C-lobe of porcine transferrin and are necessary in the utilisation of iron from transferrin as a sole source. TbpB has also been shown to be able to bind hemoglobin (18). ExbB and exbD are linked to the TbpA and TbpB proteins and form an inner membrane complex in association with TonB which enables transport of iron across the outer membrane by providing energy to high affinity receptors (106). This mechanism is of great importance in virulence as is shown by *exbB* and *tonB* mutants which are avirulent (18).

6.6.3 Heme Compounds

All serotypes of *A. pleuropneumoniae* are known to use free heme, hemin, hematin and haemoglobin. Heme is first bound by TonB-dependent heme/hemoglobin receptors and is then transported into the periplasm. Archambault *et al.* suggested that *A. pleuropneumoniae* synthesizes potential hemin and hemoglobin-binding proteins that could be implicated in the iron uptake from porcine hemin and hemoglobin and that iron-restriction increases expression of *A. pleuropneumoniae* hemoglobin receptors (4, 6). HgbA, an outer membrane protein present in *A. pleuropneumoniae*, has been suggested to play a major role in heme acquisition. Indeed, it interacts with hemoglobin to import heme into the cell. The sequence Asp592 to Pro807 of HgbA contributes to heme transport following hemoglobin binding to HgbA. It has been shown that HgbA may be self-sufficient as hemoglobin receptor, but it may also function simultaneously with another protein to

mediate heme import (100). An HgbA model has been produced by comparative modeling and by a Hidden Markov Model and showed a globular N-terminal cork domain contained within a 22-stranded beta barrel domain. This model implicates loop 2 and 7 in recognition and binding of hemoglobin or the heme ligand (84). Lipopolysaccharides and outer membrane proteins have also been suggested to bind porcine hemoglobin, but it is yet unknown if they play a role in iron acquisition (5, 6, 10, 18).

6.6.4 Transport across the cytoplasmic membrane

After iron has crossed the outer membrane, it is transported across the cytoplasmic membrane with the help of a periplasmic binding-protein-dependent transport system encoded by the *afuCDBA* operon (18, 73).
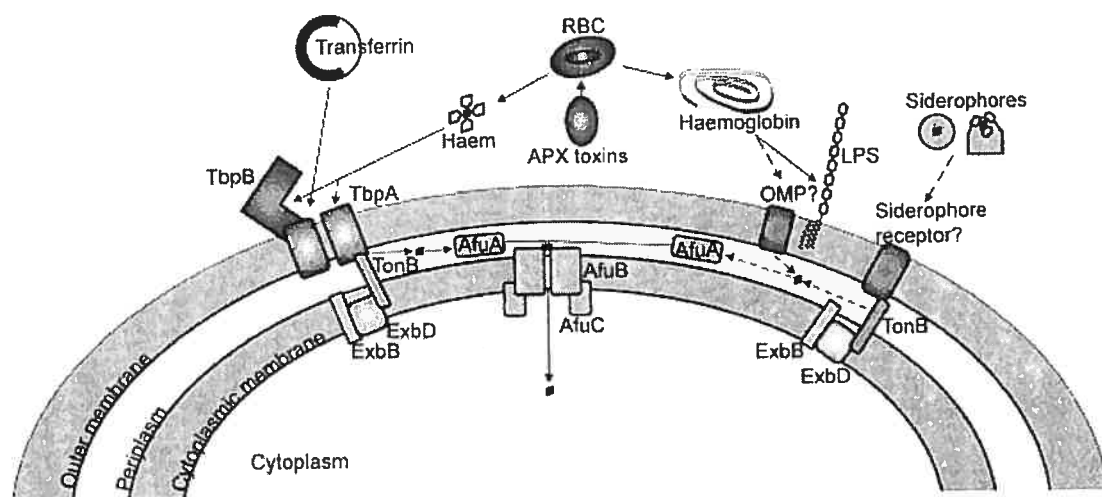
Figure 6: Diagrammatic representation of the iron uptake systems of *A. pleuropneumoniae*. Adapted from *Bossé et al.* (2002).

6.7 Other virulence factors

Other virulence factors of *A. pleuropneumoniae* include hemagglutins, as well as the secretion of a hemolysin, an urease, proteases and a superoxide dismutase. These different factors are detailed below.

6.7.1 Hemagglutinins

Different strains of *A. pleuropneumoniae* have shown hemagglutinating properties although no hemagglutinins have been described to date. In fact, seven different patterns were found depending on the origin of the agglutinated erythrocytes, though no correlation was found between the patterns of agglutination and the serotypes. Fimbriae and hydrophobic interactions are not believed to play a role in hemagglutination (56).

6.7.2 Hemolysin

The hemolysin of *A. pleuropneumoniae*, named HlyX, contains four iron-sulfur clusters (42, 99). The use of a *hlyX* mutant showed that, under anoxic conditions, the HlyX protein is responsible for the upregulated expression of both DMSO reductase and aspartate ammonia lyase. Furthermore, an hlyX mutant showed a reduction in virulence compared to the parent strain (8).

6.7.3 Proteases

A. pleuropneumoniae has the ability to secrete proteases which can degrade porcine gelatine, IgA, actin, hemoglobin and more (43). Metalloproteases have been shown to be responsible for the degradation of porcine IgA and IgG. One of the metalloprotease observed is a multimeric protein of appromaximately 101 kDa which can resist to heat and chemical denaturation (41). Another metalloprotease of more than 200 kDa was also observed in all serotypes of *A. pleuropneumoniae* (40). The role of these proteases in pathogenesis is not yet certain but it is speculated that the cleavage of IgA could facilitate the spread of *A. pleuropneumoniae* through mucus while the cleavage of hemoglobin could be an iron acquisition system of *A. pleuropneumoniae* (43).

6.7.4 Superoxide Dismutase

Copper-zinc superoxide dismutases are widely found in Gram-negative bacteria including in *A. pleuropneumoniae* (64). Superoxide dimutases are metalloproteins that prevent the accumulation of cytotoxic oxygen and nitrogen free radicals produced by the reduction of molecular oxygen by catalyzing the dismutation of superoxide radical anion to hydrogen peroxide and oxygen (96). The *A. pleuropneumoniae* superoxide dismutase is encoded by the *sodC* gene and produces a periplasmic protein (64). A *sodC* mutant of *A. pleuropneumoniae* serotype 1 created by Sheehan *et al.* showed no decrease in virulence and created lesions which were indistinguishable from those of the wild type. This may be due to the presence of the potent Apx toxins which are highly cytotoxic (96).

6.7.5 Urease

An urease enzyme, encoded by the operon *ureABC*, has been identified in *A. pleuropneumoniae* and is formed of 3 subunits (19). This operon is believed to be regulated but this assertion still remains to be determined (17). Urease-negative mutants were used in *in vivo* studies and were not able to cause infection. This indicates that urease activity may be needed for infection and that urease is indeed a virulence factor of *A. pleuropneumoniae* (20). Conversely, contradicting results were published by Baltes *et al.* who observed that an *ureC* mutant was still able to create infections undistinguishable from those of the parent strain. In addition, although the mutant could not be isolated from healthy lung tissues like the parent strain three weeks postinfection, higher number of *A. pleuropneumoniae*-specific B cells were found in the bronchoalveolar lavage fluid of pigs infected with the mutant (9). Clearly, more studies have to be done on the *A. pleuropneumoniae* urease to demonstrate its role in pathogenesis.

7. Experimental models

Many experimental models have been used to study the infection mechanisms of *A. pleuropneumoniae* including models using primary cell or tissues as well as *in vivo* models in pigs or models of adherence to biological substances like collagen and fibronectin (15, 29, 31, 45, 51). The studies done using these models greatly increased our knowledge of the infection mechanism of *A. pleuropneumoniae*. Indeed, LPS as adhesins as well as all the putative adhesins of *A. pleuropneumoniae* were found using these models (see section 6.5). The effect of different growth

conditions on adherence has also been elucidated using these models. In the present literature, there is no report of infection models using immortalized cell lines and the first report of the establishment of porcine respiratory tract cell lines dates of October 2001 (94). Two porcine respiratory tract epithelial cell lines have now been established and reported in the literature, namely the Newborn Pig Trachea (NPTr) and the St-Jude Porcine Lung (SJPL) cell lines. The NPTr cell line were established from a 2-day-old piglet from a pathogen free herd while the SJPL cell line was spontaneously established from the lung of a normal 4-week old female Yorkshire pig (33, 94). The use of these cell lines has the possibility to generate a great amount of information on the infection mechanism of *A. pleuropneumoniae* as well as that of other swine respiratory tract pathogens.

8. Techniques for the identification of novel virulence factors

Many techniques have now been developed to permit the identification of virulence factors required for infection and survival of bacterial pathogens in the host. They include, IVET, STM, SCOTS and more recently microarrays. These techniques and how they have been used to study the virulence factors of *A. pleuropneumoniae* are explained below.

8.1 In vivo expression technology (IVET)

The IVET system is based on complementation of a mutated strain and uses a tandem set of *in vivo* and *in vitro* promoterless reporter to identify promoters that are turned on in the host (69, 97). Fuller *et al.* developed an IVET system for *A.*

pleuropneumoniae based on an attenuated riboflavin mutant. The mutant strain is complemented with fragments of the genome of *A. pleuropneumoniae* which are inserted upstream of luciferase and riboflavin genes. The riboflavin genes are therefore only expressed when the genomic fragment inserted contains a functional promoter, permitting survival of the bacteria in the host. Ultimately, mutants of the genes of interest have to be constructed in order to insure the role of the genes in *in vivo* survival. Using this system in an experimental infection model in swine, Fuller *et al.* have identified ten genetic loci that are specifically induced *in vivo*. Amongst these, a loci coding for LPS biosynthesis has been identified (38).

8.2 Signature-tagged mutagenesis (STM)

STM allows for screening of multiple transposon mutants by tagging each mutant in a pool with a unique DNA sequence. A screen is then performed to find mutants which are unable to survive *in vivo* and ultimately, the mutated genes are identified (37).

In *A. pleuropneumoniae*, many virulence factors have been identified through this technique. Amongst others, a gene coding for OmpA as well as numerous genes coding for LPS biosynthesis have been detected (37, 95).

8.3 Selective capture of transcribed sequences (SCOTS)

The SCOTS technique aims to identify genes that are upregulated *in vivo*. To do this, transcribed sequences of bacteria present in the host and of culture-grown bacteria are captured using biotinylated chromosomal DNA coupled to streptavidin-

coated paramagnetic beads. Using a PCR-based subtractive hybridization with the transcripts from the culture-grown bacteria, genes that are upregulated can be identified. Southern dot blot signals which are weaker or absent in the culture-specific cDNA blot compared to those of the *in vivo*-specific cDNA represent genes that are upregulated *in vivo*. This technique is very sensitive and permits the isolation of bacterial genes expressed in tissues of infected animals.

In *A. pleuropneumoniae*, SCOTS has been used to identify 46 genes transcribed by the bacteria in necrotic porcine lung tissue. Out of these 46 genes, 20 had previously been associated with virulence or *in vivo* expression either in *A. pleuropneumoniae* or in other organisms. Amongst other, an autotransporter adhesin similar to Hsf in *Haemophilus influenzae* as well as a fimbriae-like protein and an outer membrane protein similar to PomA of *Pasteurella multocida* have been shown to be upregulated *in vivo* (7).

8.4 Microarray

Sequencing of the genome of different *A. pleuropneumoniae* serotypes has enabled researchers to use DNA microarrays to detect changes in gene expression. Presently, genome sequencing projects are in progress for the three most prevalent serotypes of *A. pleuropneumoniae* in North America, namely serotypes 1, 5 and 7. The genomic sequence of serotype 5b is being completed in Dr. John Nash's laboratory at IBS-NRC (Ottawa) using the L20 strain (49). An ORF DNA microarray was constructed from these sequences. The microarray contains spots representing 2025 ORFs of the genome in duplicate as well as controls; *A. pleuropneumoniae* L20

and porcine DNA (www.ibs-isb.nrc-cnrc.gc.ca/ glycobiology/appchips_e.html). Complementary DNA obtained from bacteria grown in conditions mimicking those of the host are labelled with different fluorescent dye than cDNA from control bacteria grown in normal nutriment rich medium (green for one sample and red for the other). The labelled cDNA are then co-hybridized on the microarray and scanned in a dual laser scanner. The intensity of fluorescence of the spots is directly proportional to the expression of the gene. Spots with high intensity for host like condition-specific cDNA and low control-specific cDNA represent up-regulated genes (46).

A study using the *A. pleuropneumoniae* 5b L20 microarray has been submitted for publication and compares gene expression of *A. pleuropneumoniae* serotype 1 grown under iron-rich and iron-restricted conditions. A restriction in iron mimics in vivo conditions as iron is a scarce element in the host. Gene identified as being up-regulated in iron-restricted conditions are therefore genes that may be required for survival *in vivo* and consequently virulence factors. In this study, 92 genes were up-regulated due to this restriction. In most cases, genes involved in iron acquisition and transport were up-regulated, including *tonB1*, *exbB1*, *exbD1*, *tbpA* *hugZ* and *hgbA*. New putative iron acquisition systems were also highlighted through this study. Aside from iron acquisition systems, a gene from the Apx toxins, *apxIC*, was also observed to be up-regulated as well as a putative autotransporter similar to the Ssa1 protein of *Mannheimia haemolytica*. Many genes with unknown function were also identified and will be further characterized to show their putative role as virulence factors (27).

Virulence Factors of Other *Pasteurellaceae*

1. *Haemophilus parasuis*

H. parasuis is an important pathogen of the swine causing fatal polyserositis, Glasser's disease which is characterised by severe inflammation of serous membranes as well as meningitis, arthritis and pneumonia (71). *H. parasuis* colonizes the upper respiratory tract to cause infection, but the mechanism by which this process is conducted is poorly known (80). *H. parasuis* has been shown to adhere and invade porcine brain microvascular endothelial cells in a recent study by Vanier *et al.* (110). High levels of adhesion and invasion were observed for the serotype 5 reference strain as well as for most field strains tested. Interestingly, higher levels of invasion were observed for serotypes 4 and 5 than for other serotypes tested (110). *H. parasuis* is also able to cross the mucosal barrier and enter the blood stream to create systemic infections, but yet again the process by which this is done is not known (108). Capsular polysaccharides are thought to provide phagocytosis resistance enabling the bacteria to survive in the blood stream but this notion is controversial (80). No toxins have been detected in the 15 serotypes of *H. parasuis* but fimbriae and transferin-binding proteins are present in at least some serotypes (67). Other putative virulence factors of *H. parasuis* include lipooligosaccharides, a capsule, fimbriae-like structures and neuraminidase activity (66, 76, 80, 115).

2. *Actinobacillus suis*

A. suis is a swine pathogen associated to many clinical signs including sudden death, dyspnea, cough, fever, neurological signs, hemorrhagic lesions on the skin ears and abdomen and gross pathological lesions. *A. suis* produces toxins similar to ApxI and ApxII of *A. pleuropneumoniae* and also express different types of LPS and capsules (67). Using PCR-based STM, Ojha *et al.* was able to identify new genes necessary for *A. suis* colonization of the respiratory tract of swine. Two have a putative attachment function; OmpA and a hemagglutinin (79). The OmpA homologue in *H. influenzae* plays an important role in the initiation of *H. influenzae* infection while the *E. coli* OmpA is known to recognise many ligands on eukaryotic target molecules and is implicated in the invasion of these cells (85, 87).

3. *Pasteurella multocida*

P. multocida is the causative agent of many diseases in various species including, in swine, atrophic rhinitis by the toxigenic capsular type D strains and pneumonia by the non-toxigenic capsular type A strains (50). *P. multocida* is known as a poor colonizer and no known adhesin has been clearly identified even though the attachment of this bacterial pathogen has been studied in various models (1, 23, 24). LPS, hemagglutinins, capsular hyaluronic acid and a major outer membrane protein have all been suggested to play a role in adhesion. The capsule is also believed to be an important virulence factor of *P. multocida* but in iron-restricted conditions, as encountered *in vivo*, the bacteria are poorly capsulated (50). Capsulated isolates are more virulent in mice and piglets but the capsular material does not seem to be

involved in adherence (52). In addition to adhesion, avian strains of *P. multocida* have been shown to invade porcine and feline epithelial cells (54). Other virulence factors of *P. multocida* include phagocyte resistance, exotoxin, fimbriae and iron-regulated factors (34).

Objectives

Although a great deal of research has been done on the infection mechanisms of *A. pleuropneumoniae*, much is still left to investigate. Indeed, little is known about the adhesion mechanism of *A. pleuropneumoniae* and much is still to learn on the bacterial and cellular response to infection. The goal of the present study is to increase our knowledge of the infection mechanism of *A. pleuropneumoniae*. Using immortalized porcine respiratory tract cell line infection models, we expect to generate a large amount of information on the adhesion and invasion abilities of *A. pleuropneumoniae* and other porcine *Pasteurellaceae*. Using microarray technology, we will also investigate the change in expression of SJPL cell proteins upon contact with *A. pleuropneumoniae*.

III. MATERIAL, METHODS & RESULTS

Interactions of *Actinobacillus pleuropneumoniae* and Other *Pasteurellaceae* with Porcine Lung and Tracheal Epithelial Cells

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Abstract

We have developed *in vitro* adhesion models for the study of respiratory pathogens of swine using two newly established porcine respiratory tract cell lines, namely the Newborn Pig Trachea (NPTr) and the St. Jude Porcine Lung (SJPL) cell lines. Using these cell lines, we studied the adherence and invasion abilities of the etiological agent of porcine pleuropneumonia, *Actinobacillus pleuropneumoniae*. Our results showed that all *A. pleuropneumoniae* strains tested adhered to both cell lines while no invasion was observed. Growth of *A. pleuropneumoniae* serotype 1 under iron- or NAD-deprived conditions did not affect the adherence to both cell lines. The adherence and invasion of other porcine *Pasteurellaceae* such as *Actinobacillus suis*, *Haemophilus parasuis*, and *Pasteurella multocida* have also been studied and showed different levels and patterns of adherence as well as some invasion abilities. The analysis of proinflammatory cytokines production by both cell lines following stimulation with heat-killed *A. pleuropneumoniae* revealed an increased production of IL-8 by the NPTr cells. A protein profiling of 608 different cell signalling proteins was performed on the SJPL cells following an interaction with *A. pleuropneumoniae* and resulted in a tendency for up-regulation of proteins involved in apoptosis. Altogether, these results demonstrate the great potential and versatility of these cell lines in the study of the pathogenesis of porcine respiratory tract pathogens.

Introduction

Porcine respiratory diseases have heavily impacted the economy of the pig rearing industry worldwide (24). The study of the infectious mechanisms of the pathogens responsible for these diseases is therefore of great importance to permit the discovery of new ways to treat and prevent infections.

Actinobacillus pleuropneumoniae, exemplar of these porcine respiratory pathogens, causes porcine pleuropneumonia by colonizing the respiratory tract of swine (24). The colonization of the host is known to be the first step of infection and is initiated by the adhesion of bacteria to host cells (16). Thus, a substantial amount of research has been conducted on the adhesion mechanisms of this pathogen. Diverse models such as frozen tracheal and lung cross-sections as well as a variety of models using primary cells such as tracheal, bucal and lung epithelial cells have been used to study the adherence capacities of *A. pleuropneumoniae* (4, 8, 11, 27). These models led to many observations about the adhesion mechanisms of *A. pleuropneumoniae*. In particular, our group demonstrated that lipopolysaccharides (LPS), principally the core region, are involved in adhesion of *A. pleuropneumoniae* to frozen porcine tracheal and lung sections (3, 10, 11, 19, 21). In addition, using diverse techniques, different groups have identified many putative adhesins, including type IV fimbriae (30), a poly-beta-1,6-N-acetyl-D-glucosamine (PGA) polymer (13), as well as a few outer membrane proteins, one of which is an autotransporter similar to Hsf of *H. influenzae* (2, 6, 27). Their role in adhesion, however, has yet to be demonstrated.

Two cell lines have been established recently; the newborn pig trachea (NPTr) (7) and the St. Jude porcine lung (SJPL) (22) cell lines. The NPTr cell line was established following serial culture of primary cells of a pathogen free 2-day-old piglet, while the SJPL cell line was spontaneously established from the lung of a 4-week-old female Yorkshire pig (7, 22).

In this study, we characterized infection models using both the NPTr and the SJPL cell lines. We tested the adhesion of *A. pleuropneumoniae* in different conditions as well as its ability to invade epithelial cells. Other swine *Pasteurellaceae*, namely *Actinobacillus suis*, *Pasteurella multocida* and *Haemophilus parasuis*, have also been tested. The models were also used to evaluate epithelial cells activation by *A. pleuropneumoniae*.

Materials & Methods

Bacterial strains and culture conditions. All strains used in this study are enumerated in Table 1. The *A. pleuroneumoniae* serotype 1 reference strain S4074 was used in all tests. All *A. pleuropneumoniae* strains as well as the *P. multocida* capsular type A and D strains (Table 1) were grown in brain heart infusion (BHI) broth and/or agar (Gibco, Burlington, VT) supplemented with 15 µg/ml NAD at 37°C in 5% CO_2. The *A. suis* strain (Table 1) was grown in the same condition with the addition of 25 µg/ml nalixidic acid and 5 µg/ml chloramphenicol. Both *H. parasuis* strains (Table 1) were grown on pleuropneumonia-like organisms medium (PPLO) broth and/or agar at 37°C without CO_2.

Cell culture. The Newborn pig trachea (NPTr) cell line (Instituto zooprofilattico Sperimental, Bresceia, Italy) (7) was grown at 37°C in 5% CO_2 in Minimum essential medium (MEM) (Gibco), supplemented with 10% fetal bovine serum (FBS) (Gibco), and 1% sodium pyruvate (Gibco). The St. Jude Porcine Lung (SJPL) cell line (St. Jude Children's Hospital, Memphis, TN) (22) was grown at 37°C in 5% CO_2 in Dulbeco's modified Eagle's medium (DMEM) (Gibco), supplemented with 10% FBS, 1% sodium pyruvate and 1.5% MEM non-essential amino acids solution (Gibco).

Microscopy. Cell were seeded to semi-confluence in wells of 4-well LabTekII chamber microscopy slides (Nunc, Naperville, IL) and incubated overnight. One ml of a 2.5×10^6 CFU/ml suspension of *A. pleuronpneumoniae* S4074 was added to the wells. The slides were then incubated 2 hours at 37°C. Four washes were performed to remove non-adherent bacteria. The cells were then fixed 10 minutes in methanol and stained 30 minutes with Giemsa stain (Sigma, St. Louis, MS). Four washes were performed to remove the excess stain and the slides were left to dry. Non-infected cells were also stained as controls. Observation was done at a 100X magnification on a Leica DMR microscope.

Adherence assay. To quantify the adherence of the different strains on both cell lines, 2.5×10^5 epithelial cells were seeded into wells of 24-well tissue culture plates (Sarstedt, Numbrecht, Germany) and incubated overnight. 500 µl of an overnight culture of the bacteria to be studied was inoculated in a fresh 5 ml broth and incubated until an OD_{600nm} of 0.6 was reached. The bacteria were resuspended in the appropriate cell culture medium to a concentration of 2.5×10^6 CFU/ml. One ml of this suspension was added to each well containing the epithelial cells and the plates were incubated from 1 to 3 hours. Non-adherent bacteria were removed by rinsing the wells four times with Dubelco's phosphate-buffer saline (DPBS) buffer (Gibco). The bacteria-associated cells were released from the wells by adding 100 µl of 1X trypsin-EDTA (Gibco) and resuspended in 900 µl DPBS buffer. Serial dilutions of the samples were performed and plated on agar plates to determine the number of

bacteria that adhered to the epithelial cells. Extra wells were used to perform bacterial counts at every incubation time in order to track bacterial growth.

Iron- and NAD-deprived adherence assay. To study the influence of iron restriction on adherence, tests were performed with *A. pleuropneumoniae* grown in BHI broth containing 50 µg/ml of ethylenediamine-N,N'-bis(2-hydrosyphenylacetic acid) (EDDHA) as well as with *A. pleuropneumoniae* grown in iron-rich conditions (no EDDHA). To study the influence of NAD restriction on adherence, tests were performed with *A. pleuropneumoniae* grown in BHI broth containing only 0.15 µg/ml NAD, as well as with *A. pleuropneumoniae* grown in NAD-rich conditions (15 µg/ml NAD).

Invasion assay. 2.5×10^5 epithelial cells were seeded into wells of 24-well tissue culture plates and incubated overnight. One ml of a 2.5×10^7 CFU/ml bacterial suspension was added to each well containing the epithelial cells and in wells containing no epithelial cells as negative controls. Plates were incubated for 1 to 3 hours. Nonadherent bacteria were removed by rinsing the wells four times with DPBS buffer. To remove any extracellular bacteria, 1 ml of culture medium containing 100 µg/ml of gentamicin and 100 µg/ml of penicillin/streptomycin was added to each well followed by a 1 hour incubation period at 37°C in 5% CO_2. Killed bacteria were removed by rinsing the wells two times with DPBS buffer. Cells were then lysed by adding 100 µl of sterile dH_2O. The water solution containing the lysed

cells as well as any invading bacteria was then plated on agar plates and incubated overnight to determine the total number of bacteria that invaded the epithelial cells.

Stimulation of cytokine production. Induction assays were performed with both cell lines as described by Ramjeet *et al.* (20). Briefly, 1 ml culture medium containing 1×10^9 *A. pleuropneumoniae* S4074, killed by a heat treatment at 60°C for 45 min, was added to wells of a 24-well tissue culture plates containing a monolayer of epithelial cells. The plates were incubated from 30 min to 48 hrs at 37°C in 5% CO_2. The supernatant was then collected and analyzed by ELISA to detect the amount of IL-1β, TNF-α, IL-6 and IL-8 produced by the stimulated epithelial cells. ELISAs were performed using the same technique and antibodies as described by Ramjeet *et al.* (20). The stimulation tests were also performed using 35 to 3500 endotoxin units/ml of *A. pleuropneumoniae* serotype 1 S4074 LPS. These LPS molecules were shown to induce a response in porcine alveolar macrophages (20).

Protein profiling of SJPL cells in contact with *A. pleuropneumoniae*. Two 175 cm^2 tissue culture flasks were seeded with a confluent monolayer of SJPL cells. 500 µl of an overnight culture of *A. pleuropneumoniae* S4074 was used to inoculate a fresh 5 ml broth which was incubated until an OD_{600nm} of 0.6 was reached. 25 ml of DMEM culture medium with or without 1×10^7 CFU/ml of bacteria was added to the flasks. Both flasks were then incubated 3 hours at 37°C in 5% CO_2. Following incubation, the flasks were washed 3 times with DPBS and 500 µl of a lysis solution containing 20 mM MOPS, 0.5% triton and inhibitors was added. Using cell

scrappers, the cells were removed from the flasks and put into microcentrifuge tubes on ice. Sonication treatments equalling ~180 joules were performed using a Cole Parmer ultrasonic processor in order to lyse the cells. The samples were then ultracentrifugated at 50,000 rpm for 30 minutes using a Sorvall RC M100 ultracentrifuge. The supernatant was conserved and analyzed for protein concentration using the Bradford assay (Bio-Rad, Hercules, CA). Samples were diluted to 2 mg/ml and frozen at -80°C. The samples were then analyzed using an antibody microarray that tracks changes in protein expression of 608 different cell signaling proteins in duplicate including phospho-sites and kinases (Kinexus, Vancouver, BC). The samples were differentially labelled and hybridized side-by-side on the same microarray. Qualitative and semi-quantitative analyzes of the expression and phosphorylation states of the cell signaling proteins were performed.

Cytotoxicity assay. The cellular cytotoxicity was measured in the different assays using the LDH measurement assay CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI). Briefly, after each incubation time, supernatants to be studied were recuperated to evaluate the release of LDH by the epithelial cells. Supernatant of non-infected cell was used as a negative, while total lysis of cells by a treatment with 2% triton was used as a 100% cytotoxicity positive control. The samples were read in a Power Wave X340 (Biotek Instruments Inc, Winooski, VT, USA) microplate reader at a wavelength of 490 nm. The results were used to calculate the percentage of cytotoxicity.

Statistical analysis. Statistical analyzes were performed using a general linear model, an extension of the ANOVA models. P<0.005 was considered statistically different.

Results

Adherence and invasion of *A. pleuropneumoniae* and other *Pasteurellaceae* to the SJPL and NPTr cell lines. Microscopy of the cell lines in the presence of *A. pleuropneumoniae* was performed to detect any interaction between *A. pleuropneumoniae* and the cells. Interactions were indeed noticed between *A. pleuropneumoniae* and both cell lines (Figure 1). The observation of the cells' morphology (Figure 1) showed that the cytoplasm of the SJPL cells is more spread, leading to a greater total size of the SJPL cells in comparison to the NPTr cells. In addition, the NPTr cells produce filamentous protrusions on which *A. pleuropneumoniae* seem to adhere preferentially (Figure 1). Standardization of the adherence model was then performed using both cell lines and the *A. pleuropneumoniae* reference strain S4074. Different MOIs (10:1, 100:1 and 1000:1) as well as different incubation times ranging from 1 to 6 hours were tested to evaluate the cytotixicity. An MOI of 10:1 and a maximum incubation time of 3 hours were used since these conditions lead to low cytotoxicity levels (Figure 2). The models were then tested using other serotypes of *A. pleuropneumoniae* as well as different swine *Pasteurellaceae*. Many differences in adherence were observed between strains for a given cell line as well as between the cell lines for a given strain (Figure 3). We noticed that the field strains of *A. pleuropneumoniae* tested adhered significantly more to the cell lines than the reference strain of the same serotype. We also noticed that the adherence level to a given cell line depends on the strain. The *A.*

suis strain H91-0380, the field *A. pleuropneumoniae* serotype 7 strain 05-3695 and the *A. pleuropneumoniae* serotype 1 strain FMV91-6514 adhered significantly more to the SJPL cell line, while the reference strain WF83 of *A. pleuropneumoniae* serotype 7 and *H. parasuis* strain 29755 adhered significantly more to the NPTr cell line. The *A. pleuropneumoniae* serotype 1 strain FMV91-6514 was less adherent to the NPTr cells in comparison to the serotype 1 reference strain S4074 but no differences were observed between these strains for the SJPL cells. The 29755 strain is much more adherent to the NPTr cells than the Nagasaki strain but no differences were observed for the SJPL cells. Invasion tests were performed on *A. pleuropneumoniae* and other *Pasteurellaceae*. *A. pleuropneumoniae* S4074 did not invade either cell lines in our infection model while other *Pasteurellaceae* showed some invasion (Data not shown).

Adherence of *A. pleuropneumoniae* serotype 1 when grown under iron-restricted or NAD-restricted conditions. Adherence tests were performed using *A. pleuropneumoniae* grown in iron- or NAD-restricted conditions to see if these conditions affect adherence. Neither conditions lead to an increase or decrease in adherence and that for both cell lines (data not shown). The cytotoxicity levels for these tests were lower than 5%.

Production of cytokines by the cells following incubation with heat-killed *A. pleuopneumoniae*. Incubations of the SJPL and NPTr cells with heat-killed *A. pleuropneumoniae* were performed to quantify the production of IL-1β, IL-6, IL-8

and TNF-α by the cells. ELISAs performed on the supernatant samples showed that the NPTr cells, but not the SJPL cells, secrete IL-8 when stimulated by heat-killed *A. pleuropneumoniae* S4074 (Figure 5). IL-8 production by the NPTr cells increased over time to reach 2500 pg/ml at 48 hours. No IL-1β, IL-6 or TNF-α were detected in the samples from both cell lines (data not shown). Purified *A. pleuropneumoniae* LPS did not induce a cellular response by either cell lines for all tested cytokines (data not shown).

Protein profiling of SJPL cells in contact with *A. pleuropneumoniae*. A protein profiling of SJPL cells in contact with *A. pleuropneumoniae* was performed using an antibody microarray in order to detect changes in protein expression of 608 cell signalling proteins, including 250 phospho-sites, 240 protein kinases and 110 cell signalling proteins that regulate cell proliferation, stress and apoptosis. Only proteins with a fold change of 1 and higher were deemed differentially expressed. Fifteen proteins were up-regulated while 24 were down-regulated (Tables 2 and 3). Amongst the up-regulated proteins, most are implicated in apoptosis and stress response. Mostly proteins implicated in cell growth and proliferation were observed to be down-regulated.

Discussion and Conclusion

Using two newly established porcine cell lines, the NPTr and SJPL, which represent respectively the upper and lower respiratory system of swine, we have developed *in vitro* models for the study of the infectious mechanism of respiratory pathogens of the swine. Indeed, we were able to study the adherence and invasion capacities of multiple *Pasteurellaceae* swine pathogens. We were also able to study the response of the cells to interactions with *A. pleuropneumoniae* by evaluating the production of cytokines and by performing protein profiling using an antibody microarray.

The adherence of the *A. pleuropneumoniae* serotype 1 reference strain S4074 was shown by microscopy. Following this observation, we used our adherence models in order to quantify the adherence and showed that all the *Pasteurellaceae* are adherent to the cell lines. Our results demonstrated that two *A. pleuropneumoniae* strains showed higher levels of adherence to the SJPL cell line while the *H. parasuis* strain 29755 adhered significantly more to the NPTr cells. This may be explained by the fact that *A. pleuropneumoniae* is known as a primarily lower respiratory tract pathogen (24) while *H. parasuis* is known as a commensal organism of the upper respiratory tract (17). Many differences were also observed between the strains for a given cell line, implicating that they possess different adhesins. The *A. pleuropneumoniae* serotype 1 strain FMV91-6514 expresses a LPS with a truncated core and no O-antigen (12) and was shown to adhere less to the NPTr cells than the

serotype 1 reference strain S4074. This is in agreement with other studies performed on tracheal cross-sections which showed that, in LPS, the core is necessary for adherence (18, 21). The observation that the field strains tested adhered significantly more to the cell lines than the reference strain of the same serotype may be explained by the fact that the field strains were recently isolated from diseased animals, while the reference strains have been repeatedly passed in standard nutrient-rich culture medium for many years, which could have lead to the lost of different surface molecules or receptors. Differences in the adherence to the NPTr cells were observed between the two *H. parasuis* strain although they are both of the serotype 5. The origin of the strains may be at cause or it could be the fact that the Nagasaki strain has been shown to have a significantly lower degree of relatedness to other *H. parasuis* strains (17).

Adhesins, as well as other virulence factors, may be expressed in conditions mimicking that of the host. Iron and NAD are scarce in the host's environment and are required by *A. pleuropneumoniae* for growth (24). For this reason, we tested the adherence of the *A. pleuropneumoniae* serotype 1 reference strain S4074 grown under iron- or NAD-deprived medium. Growth of the bacteria under iron- or NAD-deprived conditions did not change the adherence levels to either cell lines. This is in agreement with a microarray transcriptome profiling by Deslandes *et al.* in which iron-restriction did not result in up-regulation of genes coding for putative adhesins (5). It is interesting to note that a different study showed an increase in *A. pleuropneumoniae* serotype 5a, 9 and 10 adherence following NAD-restriction but no difference for the serotype 2 (27), as demonstrated here with serotype 1.

From these results, we were able to show the efficiency of our adherence models and became interested in studying the invasive capacities of different *Pasteurellaceae*. The invasion abilities of *A. pleuropneumoniae* has never been studied, while other *Pasteurellaceae,* like *Actinobacillus actinomycetemcomitans,* have been observed to be invasive (15). Here, we demonstrated that *A. pleuropneumoniae* S4074 is not invasive in our model. However, the other *Pasteurellaceae* tested, namely *A. suis, H. parasuis* Nagasaki and 29755, *P. multocida* A and *P. multocida* D, did show some invasion abilities in comparison to *A. pleuropneumoniae* serotype 1. These results are in agreement with a recent study by Vanier *et al.* in which the invasion capacities of *H. parasuis* were demonstrated using endothelial cells (28). Other studies have also shown *P. multocida* as being invasive to avian fibroblast and epithelial cells (1, 14). Our adherence and invasion results demonstrate the versatility of our models.

To further investigate the infection mechanism of *A. pleuropneumoniae* as well as the potential of our model, we studied how the cells respond to interactions with *A. pleuropneumoniae*. Purified LPS, which were shown to induce a response in porcine alveolar macrophages (20), were first used to stimulate the epithelial cells and did not result in the production of cytokines. We then used heat-killed *A. pleuropneumoniae* serotype 1 reference strain S4074, which were shown to induce higher levels of cytokine production by porcine alveolar macrophages (20), to stimulate both cell lines and evaluate the production of IL-1β, IL-6, IL-8 and TNF-α. Stimulation of the NPTr cells with heat-killed *A. pleuropneumoniae* resulted in the production of IL-8 but none of the other cytokines tested as determined by ELISA.

The same stimulation tests were performed in a study by Ramjeet *et al.* using porcine alveolar macrophages and although their results showed production of all the tested cytokines, IL-8, as observed in our model, was produced most abondantly (20). IL-8 is a chemotaxis factor for neutrophils (23), and the production of IL-8 by the epithelial cells could correlate with the high number of neutrophils in lung lesions during *A. pleuropneumoniae* infections (24). The stimulation of the SJPL cells, however, did not result in any production of the tested cytokines, as determined by ELISA. We must take into account that epithelial cells are not immune cells and thus produce lower levels of cytokine in comparison to alveolar macrophages (20). Another factor that could influence the results is the possible absence of receptors on the epithelial cells like CD14 or TLR-4, for instance, which would prohibit the response to stimulants. However, serum was present in the medium during the experiments and serum was shown to be a source of soluble CD14 (9). Furthermore, to stimulate the cells, we used killed bacteria that were obtained following a heat treatment. This could have damaged proteins necessary in adherence and could explain why some cytokines were not secreted since it is often seen that production of cytokines is stimulated by adherence of the bacteria to the cells (29).

To further analyze the response of the cells to the presence of *A. pleuropneumoniae*, we used an antibody microarray. The presence of *A. pleuropneumoniae* led to the up-regulation of 15 proteins and to the down-regulation of 24 proteins of the SJPL cells. Many of the up-regulated proteins have a direct role in apoptosis and response to stress. Apoptosis in epithelial cell may be triggered by LPS and as been shown to serve three main roles: (1) to eliminate damaged cells; (2)

to restore homeostasis following hyperplastic changes; and (3) to control inflammation, and thereby support barrier and anti-inflammatory functions (25). It is therefore not surprising that apoptosis was activated due to the presence of *A. pleuropneumoniae*, since cellular damage as well as inflammation is known to occur during porcine pleuropneumonia (24). Proteins involved in glucose and long-chain fatty acids metabolism were also up regulated most likely to create or save energy (26). In the same way, the down-regulation of cell growth proteins as well as proteins involved in protein synthesis could be due to energy saving strategies of the cells in response to the stress caused by the infection. It would also be interesting to evaluate the transriptomic profile of *A. pleuropneumoniae* following interactions with the SJPL cells to see how the bacteria respond to interactions with epithelial cells.

Overall, this study proved the efficacy and versatility of our models in the study of the pathogenesis of *A. pleuropneumoniae* and other *Pasteurellaceae*. Indeed, different levels of adherence were observed between the strains and between the cell lines. The models were also efficient to test for the invasion capacities of different swine respiratory pathogens as well as for the study of the cellular response to interaction with *A. pleuropneumoniae*. By using cell lines, we avoid the need for live animals and cell lines are not subjected to variation between individuals as primary cells do. These models are affordable and represent great tools for studying bacterial and viral respiratory tract pathogens of the swine, alone or in co-infection, as they represent both the upper and lower respiratory system of pigs.

Acknowledgement

Table 1. Bacterial strains used in the present study

Strains	Serotype	Source or reference
A. pleuropneumoniae S4074	1	K.R. Mittal[a]
A. pleuropneumoniae L20	5b	K.R. Mittal[a]
A. pleuropneumoniae WF83	7	K.R. Mittal[a]
A. pleuropneumoniae FMV91-6514	1 (Rough)	11
A. pleuropneumoniae 05-4817	5a	K.R. Mittal[a]
A. pleuropneumoinae 05-6501	5b	K.R. Mittal[a]
A. pleuropneumoniae 05-3695	7	K.R. Mittal[a]
H. parasuis Nagasaki	5	M. Gottschalk[a]
H. parasuis 29755	5	E. Thacker[b]
A. suis H91-0380	O2/K2	J. MacInnes[c]
P. multocida 88-761	A	K.R. Mittal[a]
P. multocida 1703	D	K.R. Mittal[a]

[a] Faculté de médecine vétérinaire, Université de Montréal.
[b] Faculty of Veterinary Medicine, Iowa State University.
[c] Departement of Pathobiology, University of Guelph.
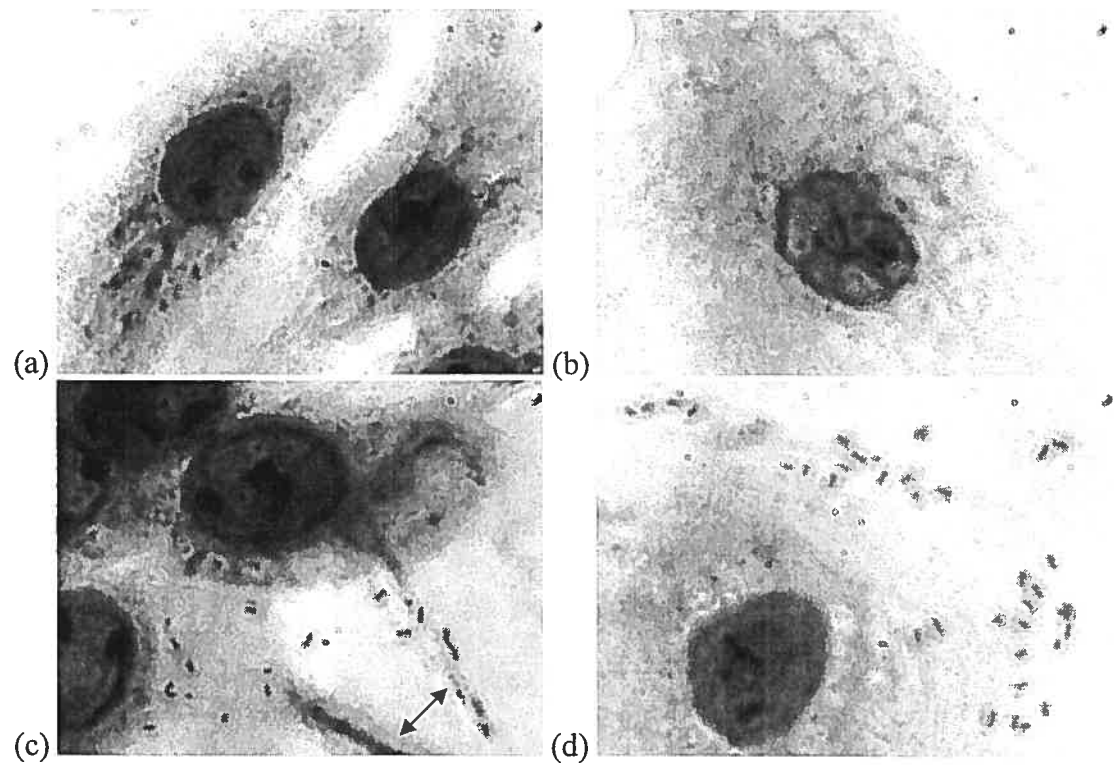
Figure 1. NPTr (a and c) SJPL (b and d) cells stained with Giemsa stain in the presence (c and d) or absence (a and b) of *A. pleuropneumoniae* serotype 1 S4074 seen through a Leica DMR microscope at a magnification of 100X. Protrusions are indicated by black arrow.
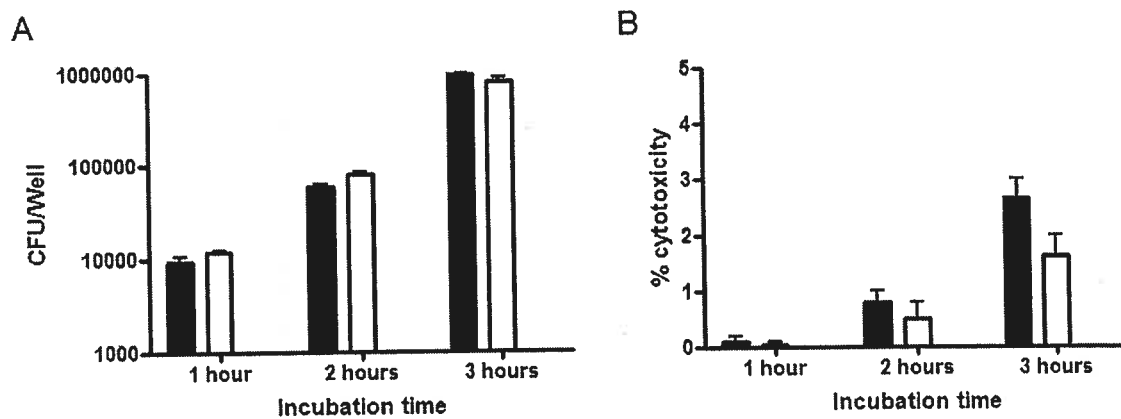
Figure 2. (A) Adherence of *A. pleuropneumoniae* serotype 1 S4074 to SJPL (filled bars) and NPTr (empty bars) from 1 to 3 hours and (B) % cytotoxicity of SJPL (filled bars) and NPTr (empty bars) following infection with *A. pleuropneumoniae* serotype 1 S4074 from 1 to 3 hours.
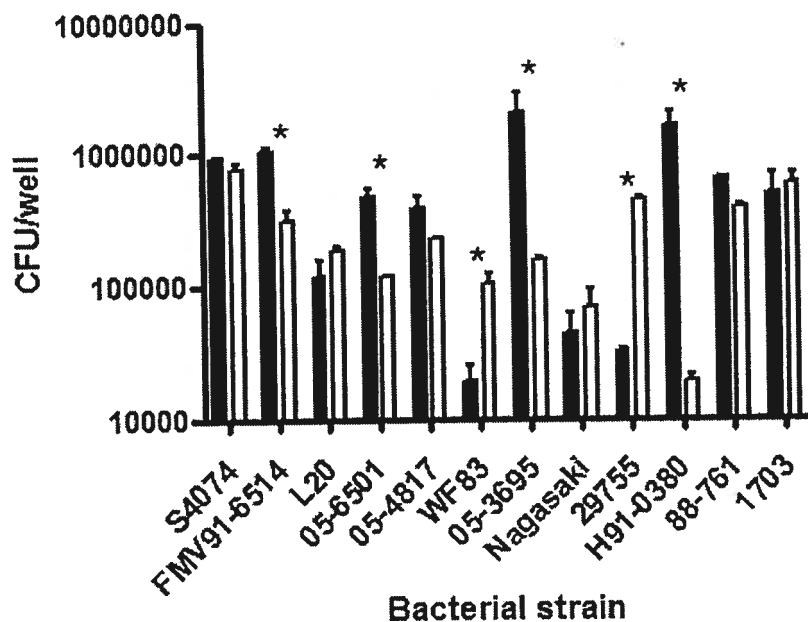
Figure 3. Adherence of twelve *Pasteurellaceae* to the SJPL (filled bars) and NPTr (empty bars) cell line after 2h of incubation. The strains include *A. pleuropneumoniae* serotype 1 S4074 and FMV91-6514, *A. pleuropneumoniae* serotype 5b L20 and 05-6501, *A. pleuropneumoniae* serotype 5a 05-4817, *A. pleuropneumoniae* serotype 7 WF83 and 05-4817, *H. parasuis* serotype 5 Nagasaki and 29755, *A. suis* serotype O2/K2 H91-0380 and *P. multocida* capsular type A 88-761 and capsular type D 1703. Asterisks represent statistical differences (P<0.05) in adherence of the given strain between the two cell line.
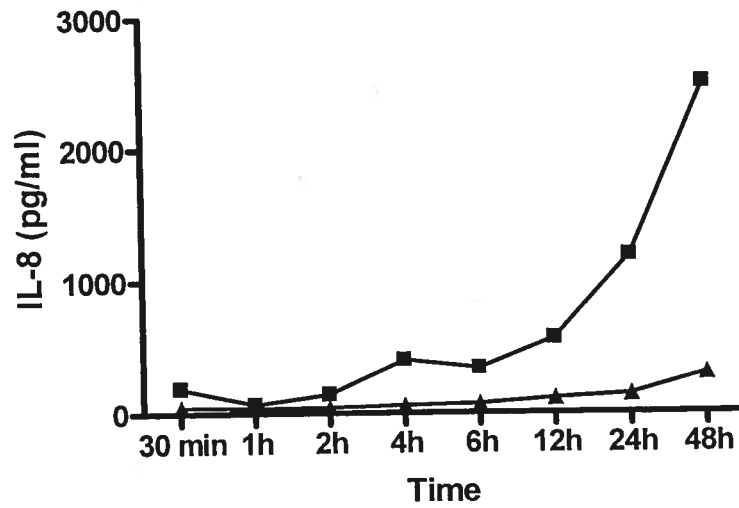
Figure 4. Production of IL-8 by NPTr cells following an induction with killed *A. pleuropneumoniae* serotype 1 S4074 (■) and when not stimulated (▲).

Table 2. Proteins of SJPL cells that are up-regulated following an infection with *A. pleuropneumoniae* serotype 1 S4074.

Swiss-prot ID	Protein	Description	Fold Change
O43293	ZIPK	ZIP kinase (death associated protein-serine kinase 3 (DAPK3))	2.04
Q9HBH9	Mnk2	MAP kinase-interacting protein-serine kinase 2 (calmodulin-activated)	2,31
Q92918	Hpk1	Hematopoetic progenitor protein-serine kinase 1	1.83
P49841	GSK3a/b	Glycogen synthase-serine kinase 3 alpha/beta	1.75
P05412	Jun	Jun proto-oncogene-encoded AP1 transcription factor	1.62
P10636	Tau	Microtubule-associated protein tau	1.38
Q15119	PyDK2	Pyruvate dehydrogenase kinase isoform 2	1.26
P42226	STAT6	Signal transducer and activator of transcription 6	1.24
P31323	PKA R2b	cAMP-dependent protein-serine kinase regulatory type 2 subunit beta	1.24
P19525	PKR1	Double stranded RNA dependent protein-serine kinase	1.20
P10809	Hsp60 (myobact-Hsp65)	Heat shock 60 kDa protein 1 (chaperonin, CPN60)	1.15
Q13085	AcCoA carboxylase	Acetyl coenzyme A carboxylase	1.11
Q07820	Mcl1	Myeloid cell leukemia differentiation protein 1	1.03

P49137	MAPKAPK2	Mitogen-activated protein kinase-activated protein kinase 2	1.02
Q14790	CASP8	Pro-caspase 8 (ICE-like apoptotic protease 5 (ICE-LAP5), Mch5, FLICE, CAP4)	1.00

Table 3. Proteins of SJPL cells that are down-regulated following an infection with
A. pleuropneumoniae serotype 1 S4074.

Swiss-prot ID	Protein	Description	Fold Change
P00519	Abl	Abelson proto-encoded protein-tyrosine kinase	-3.18
O14920	IKKb	Inhibitor of NF-kappa-B protein-serine kinase beta	-2.98
P05412	Jun	Jun proto-oncogene-encoded AP1 transcription factor	-2.34
Q15119	PDK2	3-phosphoinositide-depedent protein-serine kinase 2	-2.01
Q05209	PTP-PEST	Protein-tyrosine phosphatase with PEST sequences (PTPG1, PTPN12)	-1.99
Q13144	eIF2Be	Eukaryotic translation initiation factor 2B epsilon	-1.92
P52333	JAK3	Janus protein-tyrosine kinase 3	-1.83
P23443	S6Ka	p70 ribosomal protein-serine S6 kinase alpha	-1.76
P00533	EGFR	Epidermal growth factor receptor-tyrosine kinase	-1.51
P49841	GSK3a/b	Glycogen synthase-serine kinase 3 alpha ½	-1.49
Q13131	AMPKa1/2	AMP-activated protein-serine S6 kinase alpha	-1.45
Q02156	PKCe	Protein-serine kinase C epsilon	-1.41
NA	PI3K p110 delta	Phosphatidylinositol-4,5-biphosphate 3-kinsae catalytic subunit delta isoform	-1.40

P17252	PKCa/b2	Protein-serine kinase C alpha/beta 2	-1.39
P51636	Caveolin 2	Caveolin 2	-1.25
Q8IVT5	Ksr1	Protein-serine kinase suppressor of Ras 1	-1.14
P05556	Integrin b1	Integrin beta 1 (fibronectin receptor beta subunit, CD29 antigen)	-1.13
P09619	PDGFRb	Platelet-derived growth factor receptor kinase beta	-1.12
Q02750	MEK1	MAPK/ERK protein-serine kinase 1 (MKK1)	-1.11
O00418	eEF2K	Elongation factor-2 protein-serine kinase	-1.07
P45983	JNK	Jun N-terminus protein-serine kinase (stress-activated protein kinase (SAPK)) 1/2/3	-1.06
Q9NR28	Smac/DIABLO	Second mitochondria-derived activator of caspase	-1.06
P05771	PKCb2	Protein-serine kinase C beta 2	-1.02
P30101	ERP57	ER protein 57 kDa (protein disulfide isomerase-associated 3; 58 kDa glucose regulated protein)	-1.02

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IV. DISCUSSION

The pathogenesis of most bacteria, including *A. pleuropneumoniae*, begins with colonization. In this step, the adhesins of the bacteria bind to receptors on the host cell (78). Many models have been created to study this particular step in the pathogenesis of *A. pleuropneumoniae*. Some researchers used *in vivo* models in pigs, or organ cultures like tracheal rings or lung cross-sections while other used primary cell lines, like buccal or alveolar primary cells (15, 29, 45, 51, 109). All the models consisted of bacterial counts effectuated by microscopy. The *in vivo* models consisted of microscopic analysis of lung tissues of intranasaly infected pigs while the organ cultures and primary cells models consisted of microscopic analysis of cells and tissues infected experimentally in flasks (15, 29, 45, 51, 109). More details on the adherence mechanism of *A. pleuropneumoniae* have been discovered using these models. Our laboratory has discovered the only characterized adhesin of *A. pleuropneumoniae*: the LPS, particularly the core region (11, 48, 51, 82, 90). Using these models and other techniques, other researchers have discovered more putative adhesins. A type IV fimbriae (114) has been observed as well as a poly-beta-1,6-N-acetyl-d-glucosamine (PGA) polymer (62) and a few outer membrane proteins, one of which is an autotransporter similar to Hsf of *H. influenzae* (7, 31, 109). The role of these putative adhesins in the adherence of *A. pleuropneumoniae* is still to be confirmed. Evidently, more research has to be done on the adhesion mechanism of *A. pleuropneumoniae* in order to better understand its pathogenesis and to find ways to treat and prevent porcine pleuropneumonia.

To the best of our knowledge, no models using immortalized cell lines have been used to study the pathogenesis of *A. pleuropneumoniae*. These models are known to

be very effective and advantageous compared to other models since the cells can be routinely grown and are all virtually identical preventing discrepancies between experiments. They may, however, have undertaken some surface changes during immortalization which may affect cellular receptors (59). Two newly established cell lines representing the upper and lower respiratory tract of swine are now available; namely the Newborn Pig Trachea (NPTr) (33) and the St. Jude Porcine Lung (SJPL) (94) cell lines. Consequently, the goal of this study was to standardize adherence model using both cell lines in order to study the adherence and invasive abilities of *A. pleuropneumoniae* and other *Pasteurellaceae* as well as to study the effects that *A. pleuropneumoniae* have on the epithelial cells.

Before creating the model, we ensured, through microscopy experiments, that *A. pleuropneumoniae* interacted with the epithelial cells. These experiments showed that *A. pleuropneumoniae* did in fact adhere to both types of cells. We were also able to see the morphology of the cells; the SJPL cells are much bigger as their cytoplasm spreads more than the NPTr cells cytoplasm. We then went on to the standardization of the adherence model using the *A. pleuropneumoniae* serotype 1 reference strain S4074. This strain secretes very potent Apx toxins and as a result we had to take in account the cytotoxicity during the adherence experiments. We concluded that the use of an M.O.I. of 10:1 (bacteria:epithelial cell) as well as a maximum incubation time of 3 hours in order to yield good adherence levels with less than 5% cytotoxicity. We then tested the adherence of other serotypes of *A. pleuropneumoniae*, obtained from reference and field strains, and other porcine *Pasteurellaceae* including *H. parasuis*, *A. suis* and *P. multocida*. These experiments

were performed to ensure to efficacy or our model in studying swine pathogens in general. Our results indicate differences in adherence between cell lines and between strains. We noticed strains that are known to be more specific to the lower respiratory tract like some *A. pleuropneumoniae* and *A. suis* strains which adhered more to the to SJPL cells. This is in agreement with the fact that *A. pleuropneumoniae* is pirmarly know as a lower respiratory tract pathogen (103). The *H. parasuis* strain, however, was observed to be more adherent to the NPTr cells. This may be explained by the fact that *H. parasuis* is a commensal organism of the upper respiratory tract (80). Many differences were also observed between the strains for a given cell line, implicating that they posses different adhesins. The *A. pleuropneumoniae* serotype 1 strain FMV91-6514 expresses a LPS with a truncated core and no O-antigen (53) and was shown to adhere less to the NPTr cells than the serotype 1 reference strain S4074. This is in agreement with other studies performed on tracheal cross-sections which showed that, in LPS, the core is necessary for adherence (51, 81). For *A. pleuropneumoniae*, we noticed that the field strains adhere more to the cell lines than the reference strain of the same serotype. This is probably due to the fact that the field strains were recently isolated from diseased animals while the reference strains have been repeatedly passed in culture medium for many years which could have lead to the lost of diferrent surface molecules or receptors. For *H. parasuis*, we noticed differences between the two strains tested even though they are of the same serotype. This may be due to the origin of the strains or to the fact that the Nagasaki strain is known to be especially different than other *H. parasuis* strains (80).

Following these observations, we could conclude that our model was efficient to study the adherence mechanism of *A. pleuropneumoniae* and other swine pathogens. Iron and NAD are scarce elements in the host and essential for *A. pleuropneumoniae* growth (103). A lack of these elements may therefore trigger the overexpression of virulence factors such as adhesins. To investigate this hypothesis, we tested the adherence of *A. pleuropneumoniae* grown in iron- or NAD-restricted conditions. Neither condition lead to an increase in adherence suggesting that no adhesins that recognise receptors on the cells were overexpressed. This is in agreement with a microarray transcriptome profiling by Deslandes *et al.* in which iron-restriction did not result in up-regulation of genes coding for putative adhesins (27). A different study, however, showed an increase in *A. pleuropneumoniae* serotype 5a, 9 and 10 adherence following NAD-restriction. In the same study *A. pleuropneumoniae* serotype 2 did not show a change in adherence following NAD restriction (109).

A. pleuropneumoniae has never been shown to be invasive but other *Pasteurellaceae* like *A. actinomycetemcomitans* have (72). Due to this information, we were interested to see if *A. pleuropneumoniae* could invade the NPTr and SJPL cells. We found that *A. pleuropneumoniae* serotype 1 does not invade the cells at an MOI of 10:1 or 100:1. We then compared the invasive capacities of the other *Pasteurellaceae;* namely *H. parsuis, A.suis* and *P. multocida.* We noticed that they all had higher invasive capacities than *A. pleuropneumoniae.* These later tests were performed at an MOI of 100:1. Although leading to higher cytotoxicity, this MOI lead to better bacterial counts. The results for *H. parasuis* are in agreement with a recent study by Vanier *et al.* in which the invasive capacities of this pathogen to

endothelial cells were demonstrated (110). The invasion of avian fibroblast and epithelial cells by *P. multocida* was also previously demonstrated (2, 65).

Using our models, we were able to gather information on *A. pleuropneumoniae* and the other *Pasteurellacea*. But we questioned ourselves on how the cells respond to the presence of the bacteria. To investigate this question, we performed inductions to measure the production of pro-inflammatory cytokines. It is known that during infection, pro-inflammatory cytokines, including IL-1β, IL-8 and TNF-α, are detected in alveolar fluid and tissue lesions (103). We first started using LPS for the stimulation of cytokine production, but we soon apprehended that no response was induced. These LPS were previously shown to stimulate porcine alveolar macrophages, demonstrating their potency. We could not use live *A. pleuropneumoniae* due to the Apx toxins and the length of incubation times. We thus used heat-killed *A. pleuropneumoniae*. The killed bacteria gave us better results with a production of IL-8 by the NPTr cells. No other cytokine tested were produced by the NPTr cells and no cytokines were released by the SJPL cells. The same experiments were performed in a study by Ramjeet *et al.* in which they used alveolar macrophages. In that study, IL-8 was also the most produced although they did have release of all cytokines tested (86). We must take in account that epithelial cells are not immune cells and that the SJPL cells are in an environment where numerous immune cells like alveolar macrophages are present (26). Another possible reason for this lack of interleukin secretion may be the possibility that some receptors like CD14 or TLR-4 are missing. It is important to note however, that serum was present in the medium during the experiments which is known to be a source of soluble CD14 (47).

In addition, the heat treatment received by the bacteria may have damaged proteins involved in adherence, a process which has previously been shown to be, at times, necessary for cytokine production (113). Since IL-8 is a chemotaxis factor for neutrophils, its production by the cells agrees with the fact that a magnitude of neutrophils accumulate at the site of infection during porcine pleuroneumonia (98, 103).

To further investigate the cellular response to the presence of *A. pleuropneumoniae*, we used an antibody microarray which screens for 608 differnet cell signalling proteins. Cell lysate of SJPL cells in contact with *A. pleuropneumoniae* serotype 1 and SJPL cells in sterile culture medium were compared side-by-side on the same microarray. The results show a tendency for the up-regulation of proteins implicated in apoptosis and for the down-regulation of genes involved in cell growth and proliferation. Apoptosis in epithelial cell may be triggered by LPS and as been shown to serve many roles including the elimination of damaged cells, the restoration of homeostasis following hyperplastic changes, and the control of inflammation, and thereby the support of the barrier and anti-inflammatory functions (105). It is therefore not surprising that apoptosis was activated due to the presence of *A. pleuropneumoniae*, since cellular damage, as well as inflammation, is known to occur during porcine pleuropneumonia (103). From the light of these results, it is in our future plans to perform apoptosis tests on both cell lines.

In perspective, we are also interested in further studying the response of the bacteria upon adherence to the cell lines. For this, we plan on using DNA microarray technology to compare the gene expression of *A. pleuropneumoniae* in contact with the cells with *A. pleuropneumoniae* in sterile culture medium.

V. CONCLUSION

In conclusion, using our models, we were able to show that *A. pleuropneumoniae* and other *Pasteurellaceae* adhere to both the NPTr and the SJPL cell lines, that the adherence of *A. pleuropneumonie* is not affected by an iron or NAD restriction and that *A. pleuropneumoniae* does not invade the cells while the other *Pasteurellaceae* have this capacity. We were also able to show how the epithelial cells respond to the presence of *A. pleuropneumoniae*. The NPTr cells are stimulated to release IL-8 but none of the other cytokines tested. The SJPL cells seem to overexpress proteins involved in apoptosis and underexpress proteins involved in cell growth and proliferation.

Overall, these results show the efficacy and versatility of our models, which represent both the upper and lower respiratory tract of swine, as well as the usefulness of these cell lines in the study of bacterial respiratory pathogen of the swine.

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APPENDIX 1

Title of Conference Presentations and Posters

ASM Conference on Pasteurellacea, Kohala Coast, HI, October 2005.

Poster and oral presentation:

Transcriptional Profiling in *Actinobacillus pleuropneumoniae* Cultivated Under Iron-Restricted Conditions

V. Deslandes, J. H. E. Nash, J. Harel, E. Auger, M. Jacques*

CRAWD, St-Louis, MO, December 2005.

Poster:

Adherence of Swine Respiratory Pathogens to Immortalised Porcine Epithelial Cell Lines

E. Auger*, M-È. Cardin, I. Gaucher, M. Gottschalck, M. Jacques

ACCORD ET PERMISSION DES COAUTEURS D'UN ARTICLE[1]

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Revue	Date de publication
Infection and Immunity	En préparation

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Déclaration
À titre de coauteurs de l'article identifié ci-dessus, nous autorisons le microfilmage du mémoire et nous sommes d'accord que Eliane Auger inclut cet article dans son mémoire de maîtrise qui a pour titre « Study of the Adherence of Actinobacillus pleuropneumoniae to immortalised porcine epithelial cells ».

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