Actinobacillus pleuropneumoniae

A Major Respiratory Pathogen in Pigs

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Abstract

Actinobacillus pleuropneumoniae is a major cause of respiratory disease in pigs, causing animal suffering and substantial economic losses. The aim of this thesis was to obtain more knowledge on the protective immunity to infections with *A. pleuropneumoniae* and to evaluate potential strategies in preventing and combating *A. pleuropneumoniae* infections.

Investigations regarding the role of maternal immunity for the protection of the offspring and the subsequent effect on the epidemiology on herd level demonstrated that the levels of serum antibodies to *A. pleuropneumoniae* in sows were reflected in colostral levels. The colostral levels were in turn reflected in the levels of serum antibodies in the offspring and piglets with high levels of antibodies also had detectable levels of antibodies for a longer time compared to the offspring to sows with low levels of antibodies to *A. pleuropneumoniae*.

An inoculation of naïve pigs with *A. pleuropneumoniae* induced acute-phase protein responses. The response to the inoculation was clearly affected by the antimicrobial treatment administered at the onset of clinical signs of respiratory disease. The response of these pigs to a second inoculation was also influenced by the treatments carried out after the first inoculation. Enrofloxacin was superior in reducing clinical signs but left pigs unprotected at the second inoculation. Tetracycline demonstrated a similar treatment efficacy as enrofloxacin but pigs were protected at challenge as were the penicillin treated pigs. Penicillin was on the other hand not efficient in curing diseased pigs. The pigs that were protected from disease at the second inoculation had all developed serum antibodies to *A. pleuropneumoniae* following the first inoculation and an acute-phase response was not induced following the second inoculation. The results therefore indicate that antibodies mirror protection against disease well.

Vaccinations against actinobacillosis in a fattening herd did not provide protection against clinical disease. However, in combination with intensified treatments of pigs with signs of respiratory disease, pleurisy registrations at slaughter decreased over time although the growth rate was unaffected.

Keywords: pig, *Actinobacillus pleuropneumoniae,* immunity, vaccination, acute-phase proteins, serology, antibody, DWG, antimicrobials, epidemiology, PRDC

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To all four-legged friends that have crossed my path

The seemingly impossible is possible
Hans Rosling

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Sjölund, M., Zoric, M., Persson, M., Karlsson, G. & Wallgren, P. (2010). Disease patterns and immune responses in the offspring to sows with high or low antibody levels to *Actinobacillus pleuropneumoniae* serotype 2. *Research in Veterinary Science* doi: 10.1016/j.rvsc.2010.07.025.

II Sjölund, M., Martín de la Fuente, A.J., Fossum, C. & Wallgren, P. (2009). Responses of pigs to a re-challenge with *Actinobacillus pleuropneumoniae* after being treated with different antimicrobials following their initial exposure. *Veterinary Record* 164, 550-555.

III Sjölund, M., Fossum, C., Martín de la Fuente, A.J., Alava, M., Juul-Madsen, H.J., Lampreave, F., & Wallgren, P. Effects of various antimicrobial treatments on serum acute-phase responses and leukocyte counts in pigs after a primary and a secondary challenge infection with *Actinobacillus pleuropneumoniae*. (Submitted for publication).

IV Sjölund, M. & Wallgren, P. (2010). Field experience with two different vaccination strategies aiming to control infections with *Actinobacillus pleuropneumoniae* in a fattening pig herd. *Acta Veterinaria Scandinavica* 52:23.

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Abbreviations

AI	Artificial insemination
Apx	*Actinobacillus pleuropneumoniae* RTX-toxin
BALF	Bronchoalveolar lavage fluid
BALT	Bronchus associated lymphoid tissue
CPS	Capsular polysaccharide
DWG	Daily weight gain
ELISA	Enzyme linked immunosorbent assay
Ig	Immunoglobulin
IL	Interleukin
LPS	Lipopolysaccharide
MIC	Minimal inhibitory concentration
NAD	Nicotinamide adenine dinucleotide
NVI	National Veterinary Institute (SVA)
OMP	Outer membrane protein
PBS-T	Phosphate-buffered saline with Tween
PCR	Polymerase chain reaction
PCV2	Porcine circovirus type 2
PFGE	Pulsed-field gel electrophoresis
Pig-MAP	Pig Major acute-phase protein
pMBL	Porcine mannan-binding lectin
PRDC	Porcine respiratory disease complex
PRRSV	Porcine reproductive and respiratory
RTX	Repeats in the structural toxin
SAA	Serum amyloid A
SJV	Jordbruksverket/Swedish Board of Agriculture
SPF	Specific pathogen free
SVA	Statens veterinärmedicinska anstalt
TNF	Tumor necrosis factor

1 Background

As many diseases affecting pigs are dependent on the production system in which pigs are reared, a brief description of the development and the present structure of pig production in Sweden is given below. The major respiratory pathogens will also be briefly mentioned to provide a background for this thesis. As the immune system plays an important role in the pathogenesis of actinobacillosis, a brief introduction to the immune parameters mobilized in the respiratory tract of pigs infected with *Actinobacillus pleuropneumoniae* is given as well.

1.1 Pig production in Sweden

1.1.1 The Swedish pig population

Pig production has changed considerably over the years ever since domestication took place. The most dramatic changes have occurred during the last 200 years. This time period coincides with the industrial revolution when also the human population increased which in turn led to an increase in the demand for food.

The number of pigs in Sweden during the major part of the nineteenth century remained relatively constant at approximately 500 000. The pig population only began to increase during the last decade of the 1800's. After this, the number of pigs varied during the first half of the 1900's but then steadily increased until the 1980's. From 1985 when there were 2 645 797 pigs in Sweden, the highest number recorded, the number of pigs have declined with 44% to 1.5 million pigs in 2009 (SJV, 2010b).

A reduction has also been seen in the number of pig herds which has decreased with 90% from 26 122 in 1980 to only 2 380 in 2008. As the number of herds has decreased more than the number of pigs, the average herd size has consequently increased. In 1980, piglet producing herds on average had 15 sows (including boars) while this number had increased to 80 in 2009. The same pattern holds true for fattening herds which on average had 81 fattening places per herd in 1980. In 2009, this figure was 532 places per herd (SJV, 2010b). Two thirds of the total number of sows belonged to herds with 200 or more sows which only accounted for 15% or the total number of piglet producing herds.

1.1.2 Swedish animal welfare law requirements

According to the Swedish animal welfare law, the area requirement is 0.17 m^2 + (weight in kg/130 kg) m^2 for growing pigs (SJV, 2010a), which corresponds to an area of 0.4 m^2 per weaner of 30 kg and 0.94 m^2 for a market weight pig of 100 kg. Fully slatted floors are not allowed but about 30% of the pen may have slats.

Also all sows must be kept loose during all times. Normally, they are kept in groups during pregnancy and in individual pens during lactation. The use of farrowing crates is prohibited by law but individuals may be confined in crates for up to one week at parturition and insemination under special circumstances. The animal welfare law also prohibits weaning before four weeks of age, and weaning generally takes place at the age of four to five weeks.

1.1.3 The Swedish breeding system

The Swedish pig production is organized in the form of a pyramid with the nucleus herds (n=26) at the top, multiplying herds (n=42) second to the top, then piglet producing herds and fattening herds at the base (Figure 1). Piglet producing herds may also rear piglets to market weight in farrow-to-finish systems. In the piglet producing herds, Landrace (L) x Yorkshire (Y) sows, mated with either Duroc (D) or Hampshire (H) boars; give birth to three-breed crosses for the production of slaughter pigs (H-YL or D-YL).

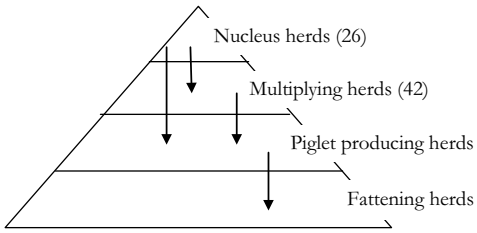

Figure 1. The breeding structure of Swedish pig production. Arrows indicate movements of animals. (Number of herds)

Traditionally, hybrid gilts (LY) have been sold from nucleus and multiplying herds to piglet producers, either as prospective gilts at a weight of 30 kg, as unmated gilts at an age of six months or as pregnant gilts at an age of 9-10 months. The piglet producers replace on average 30-40% of their breeding stock annually. Artificial insemination (AI) is used for the vast majority (>95%) of the matings; and most boars are today only used for teasing. AI has made it possible for piglet producers to recruit their own breeding stock by using two-breed rotational crossing (Y x LY; L x YLY; Y x LYLY *et. c.*). An increasing number of herds have commenced to do so during the last decade, either for biosecurity or of economical reasons (or both).

1.1.4 Production strategies

Previously, most piglets were sold from specialized piglet-producing herds to specialized fattening-herds at an approximate age of 10 to 14 weeks when weighing 25 kg. Up to the 1980's, there could be as many as 50 suppliers to one batch of approximately 400 fattening pigs. Today, growers weigh approximately 30 kg at the time of allocation to the fattening herd and they are usually between the age of nine to 12 weeks. Due to the increased herd sizes, specialized fattening herds of today usually contract two to four piglet producers in order to reduce the number of infection routes when establishing their batches. However, farrow-to-finish production systems; either within a herd, or in cooperation between herds, form an increasing part of the Swedish pig production. Further, today about 25% of the Swedish pig production takes place in fairly large (350 to 5000 sows) multi-site-systems (sow-pool-systems).

1.1.5 Health status of Swedish pigs

Sweden is located on the Scandinavian Peninsula, and together with a restricted import of live animals, the pig population has been quite isolated. Thus, the Swedish pig population has a favorable health status. Sweden is

free from all diseases listed by the Office International des Epizooties (OIE), including Aujeszky's disease (AD) and porcine reproductive and respiratory syndrome (PRRS), as well as from porcine epidemic diarrhea (PED) and transmissible gastro-enteritis (TGE). Aujeszky's Disease was diagnosed in 1963 and eradicated from Sweden in 1996 (NVI, 2010). Isolation of *Salmonella* spp. is rare, and since 1984 generally diagnosed in less than 5 pig herds per year (NVI, 2010). The few salmonella positive pigs are usually detected at slaughter and salmonella is not regarded as a clinical disease in pigs in Sweden.

Nucleus and multiplying herds in Sweden are affiliated to extended control programs and are declared free from atrophic rhinitis (toxin producing strains of *Pasteurella multocida*), *Salmonella* spp, swine dysentery (*Brachyspira hyodysenteriae*) and mange (*Sarcoptes scabei*) in addition to the above listed diseases.

In specific-pathogen free (SPF) systems, pigs are declared free from a number of defined microorganisms (Young, 1955). Pigs in the Swedish SPF system are apart from the infections mentioned above declared free from a number of major causes of respiratory infections such as PRRS-virus, *Mycoplasma hyopneumoniae*, toxin-producing *P. multocida, A. pleuropneumoniae* and swine influenza virus. The Swedish SPF system was introduced in 1988 (Wallgren & Vallgårda, 1993a), and today about 4% of the Swedish sows belong to SPF production. Production parameters such as weight gain and feed conversion ratio are greatly improved in SPF-pigs (Wallgren *et al.*, 1993b).

The organic pig production constitutes less than 1% of the total pig production in Sweden (Wallenbeck, 2009). There are no specific health qualifications for organic pig production, but the weaning age is higher (seven weeks) and the area requirements larger than for conventional pigs.

So called growth-promotors (low dose antibiotics in feed), were introduced in the 1970's, which improved production considerably, especially during the post weaning period. As the first country in the world, Sweden banned the use of growth promotors in 1986. During the subsequent years intestinal health problems of recently weaned piglets increased (Robertsson & Lundeheim, 1994), which necessitated improvements in the rearing systems in Swedish herds. Consequently, over 90% of the Swedish pigs are today

reared in batch systems based on age-segregation from birth-to-slaughter (Holmgren, 2002).

The most common diseases among pigs in Sweden are intestinal disorders in young growers and respiratory diseases in fattening pigs. In this context, it has been demonstrated that the likelihood of infection with respiratory diseases increased with increasing herd size (Moorkamp *et al.*, 2009; Maes *et al.*, 2000). Infections with *M. hyopneumoniae* and *A. pleuropneumoniae* are widespread in the conventional pig population in Sweden, but the influence of these diseases has decreased since the early 1990's (Holmgren & Lundeheim, 2002). However, during recent years problems with *A. pleuropneumoniae* have increased again (Beskow *et al.*, 2008).

1.1.6 Disease preventing measures

After the ban of the use of growth-promoters in 1986, disease prevalence increased and consequently the use of therapeutic antimicrobials also increased (Wallgren, 2009). This forced farmers, veterinarians and scientists to seek alternative solutions and improve the rearing conditions in order to improve health status. The introduction of all in-all out procedures on a large scale reduced the incidence of recordings of respiratory lesions at slaughter at a national level (Holmgren *et al.*, 1999), and today a majority of the pigs are reared in age-segregated rearing systems all the way from birth to slaughter. An improvement of the respiratory health status by implementation of all in – all out rearing has also been recorded elsewhere (Busch & Jensen, 2006; Cleveland-Nielsen *et al.*, 2002).

Other bio-security measures include restrictions for visitors, supplying visitors with protective clothes and boots, quarantine for purchased animals and refraining from introducing new pigs to the herd. Prevention of disease also includes attempts to eradicate specific pathogens, of which establishing SPF herds is the ultimate solution.

Vaccination programs have also been widely employed against common infections. In general, all sows are vaccinated against neonatal colibacillosis, parvovirus and erysipelas. Some sows are vaccinated against porcine circovirus type 2 (PCV2). Growing pigs may be vaccinated against PCV2, *M. hyopneumoniae* and *Lawsonia intracellularis*.

1.1.7 Production performance

As seen in Table 1, the productivity of the Swedish pigs varies between herds. Data on production performance is based on the data system for pig production, PigWin 2009. Based on the recordings from 72 000 sows in 186 herds, sows in mean gave birth to 12.3 piglets per litter of which 10.5 were weaned, which corresponds to a yearly production of 23.0 piglets per sow per year.

In 2009, pigs in Sweden reached market weight of 110 kg at 181 days of age while SPF pigs reach market weight at the age of 141 days. Conventional pigs required 35.4 MJ per kilo weight gain in mean while the 25% best performing herds only require 33.8 MJ. The mean daily weight gain (DWG) was 876 grams per day based on the recordings from 338 500 slaughtered pigs from 120 herds.

The mortality of piglets from birth to weaning was 17.0% and with a mortality of 2.3% during the weaning period. The mortality during the fattening period was 2.4% on average and 1.8% for the top 25% herds.

Table 1. *Swedish pig production performance in 2009 according to PigWin.*

Production parameters sows	SPF	Top 25%	Mean	Bottom 25%
Weaned piglets per sow and year	25.2	25.4	23.0	19.0
Live-born piglets per litter	13.5	13.7	12.3	10.4
Weaned piglets per litter	11.5	11.6	10.5	8.6
Mortality from birth to weaning (%)	15.5	14.8	17.0	20.7
Mortality from weaning to delivery (%)	0.1	1.6	2.3	3.6
Age at 30 kg bw (days)	70	80	83	86
Production parameters fatteners				
Daily weight gain from 30 kg bw (g/day)	1024	909	876	845
Age at slaughter (days)	141	171	181	192
Feed conversion (MJ/kg bw)	-	33.8	35.4	37.4
Mortality from delivery to slaughter (%)	0.5	1.8	2.4	3.2

1.1.8 Registrations at slaughter

Recordings made in the routine meat inspection at slaughter can be a valuable tool when evaluating the health status of pig herds over time. However, the lung lesions observed at slaughter do not provide information on the specific etiological causes of the lesions (Jirawattanapong *et al.*, 2009).

1.2 Porcine respiratory disease complex

Porcine respiratory disease complex (PRDC) is a multifactorial disease syndrome involving several respiratory pathogens. Commonly isolated bacterial pathogens are *A. pleuropneumoniae*, *M. hyopneumoniae, P. multocida, Streptococcus suis* and *Haemophilus parasuis* and common viral pathogens include porcine reproductive and respiratory syndrome virus (PRRSV), swine influenza virus (SIV), PCV2 and porcine respiratory coronavirus (Thacker, 2001). The most frequently isolated pathogens differ between countries (Hansen *et al.*, 2010; Kim *et al.*, 2003; Thacker, 2001). From an international perspective, PRRSV is one of the major pathogens (Thacker, 2001). Sweden was free from PRRS until 2007 when a total of seven production sites were infected with the European strain of the virus. These sites were stamped out and the country was again declared free from PRRS in the same year (Carlsson *et al.*, 2009). However, Sweden is currently experiencing emerging problems with *A. pleuropneumoniae* (Beskow *et al.*, 2008).

Finishing pigs between the age of 14 and 20 weeks are most commonly affected by PRCD (Thacker, 2001), but there are large variations in morbidities and mortalities between herds (Sørensen, 2006). PRDC is characterized by growth retardation; reduced feed-conversion efficiency, anorexia, fever, cough and dyspnoea. Lesions are mainly found in the anterio-cranial parts of the lungs and there is usually fibrotic pleurisy of the diaphragmatic lobes. The lesions are of course dependent on the pathogens involved.

1.2.1 Major bacterial pathogens associated with PRDC

M. hyopneumoniae is a major cause of respiratory disease in pigs, most often occurring as subclinical infections in growing and finishing pigs, causing growth retardation (Rautiainen *et al.*, 2000; Wallgren *et al.*, 1993). *M. hyopneumoniae* infections are considered to be endemic in most herds and as many as 90% of the pigs have seroconverted at the time of slaughter (Maes *et al.*, 2000; Maes *et al.*, 1999; Wallgren *et al.*, 1993). Transmission of *M. hyopneumoniae* by carrier pigs is considered to be the main source of infection under field conditions as infected pigs may harbour the microbe in the respiratory tract for up to 200 days (Pieters *et al.*, 2009). *M. hyopneumoniae* colonizes the airways by binding to cilia of epithelial cells in the respiratory tract. This results in a reduced capacity of the mucociliary apparatus to clear the respiratory tract of debris and microorganisms which

in turn paves the way for other respiratory pathogens such as *P. multocida* and *Bordetella bronchiseptica*.

Pasteurella multocida, a Gram-negative facultative anaerobic bacteria, is commonly isolated from the nasal cavity of pigs, including SPF pigs, and pneumonic pasteurellos is often considered as the common final stage of PRDC (Pijoan, 2006). There are five serogroups of *P. multocida* strains (A, B, D, E and F), classified according to their capsule antigens. Strains associated with pneumonia are rarely toxin-producing and most often they belong to serogroup A, but they may also be type D which is toxin-producing and causes atrophic rhinitis in growing pigs. *P. multocida* is often isolated from pigs primarily infected with *M. hyopneumoniae*. The chronic form of disease is most common in which occasional coughing and sometimes a slight rise in temperature is observed (Bölske *et al.*, 1980). The clinical signs are thus indistinguishable from those associated with *M. hyopneumoniae*. Varying degrees of pleuritis is also associated to *P. multocida* infections which make the lesions indistinguishable from those caused by *A. pleuropneumoniae* as seen at the post-mortem inspection at slaughter (Jirawattanapong *et al.*, 2009).

1.2.2 Major viral pathogens associated with PRDC

The two major viral infections included in the PRDC complex are SIV and PRRSV. SIV readily undergo changes in their hemagglutinin (H) and neuraminidase (N) glycoproteins causing new variants of differing virulence to arise. Infections with SIV in naïve pig herds usually cause a sudden onset of disease symptoms in a large number of animals displaying symptoms such as anorexia, lethargy and fever. Acute outbreaks of SIV are clinically indistinguishable from those of acute actinobacillosis (Tobias *et al.*, 2009). Infections with SIV have attracted more attention following the 2009 pandemic HINI-influenza among humans which was also transmitted to pigs with non-existing to mild or moderate disease symptoms (Hofshagen *et al.*, 2009).

PRRSV has become a major pathogen of most swine producing countries over the last decades. Apart from causing reproductive failure including abortions, it is also a major cause of respiratory disease in growers and fatteners. Symptoms vary depending on the route of infection and the virus strain. Concurrent infections also affect the course of disease (Hansen *et al.*, 2010).

A third virus included in the PRDC complex is PCV2. PCV2 is involved in post-weaning multisystemic wasting syndrome in growing pigs. The most prominent symptom is wasting which cause pigs to rapidly lose weight and body condition, but also an interstitial pneumonia is commonly seen histopathologically in affected pigs.

1.2.3 Parasitic pathogens associated with PRDC

A number of parasites may also be involved in the PRDC complex. The most common gastrointestinal parasite in pigs, *Ascaris suum,* may cause respiratory disease symptoms such as coughing and difficulty in breathing as larvae migrate from the intestine to the liver and then to the lung before being swallowed to finally mature in the gut. The damage to the lungs caused by the migrating larvae may pave the way for secondary bacterial invasions (Stewart, 2006).

Also lung worms (*Metastrongylus* spp.) causes lung damage under larval migration. This may induce coughing in itself, but may also facilitate subsequent infections preferably caused by common bacterial infections, such as *M. hyopneumoniae*, *A. pleuropneumoniae, P. multocida* and *S. suis* (Stewart, 2006).

1.3 Actinobacillus pleuropneumoniae

A. pleuropneumoniae belongs to the family *Pasteurellaceae* (VetBact, 2010; Brenner, 2005), and is known to cause disease in a number of species including humans. In bovines, *Actinobacillus lignieresii* is the cause of actinomycosis (wooden tongue) which was first accounted for in 1902 from subcutaneous abscesses in the head and neck region of Argentinean cattle (Rycroft & Garside, 2000). In humans, *Actinobacillus actinomycetemcomitans* causes periodontal disease (Henderson *et al.*, 2002) and in horses *Actinobacillus equuli* is the cause of fatal septicaemia in foals (Sternberg, 1999).

A. pleuropneumoniae, formerly *Haemophilus pleuropneumoniae, H. parahaemolyticus,* is a small to medium-sized, haemolytic, facultative anaerobic, Gram-negative, encapsulated rod. One complete genome sequence of *A. pleuropneumoniae* has been published (strain L20 of serotype 5b, GenBank accession number CP000569). The genome is 2.3 Mbp in length. No plasmid was found in the sequenced strain of the species (Foote *et al.*, 2008). For cultivation, *A. pleuropneumoniae* requires either special media such as chocolate agar and pleuropneumoniae-like organism agar

(PPLO); or the supplementation with nicotinamide adenine dinucleotide (NAD) or a staphylococcal streak on blood agar for it to grow. Bacteria can be cultivated from acutely affected lung tissue, lung abscesses and tonsils but overgrowth by other bacteria is common. *A. pleuropneumoniae* is rarely isolated from chronic lesions, but demonstration of *A. pleuropneumoniae* DNA by PCR can be made in subclinically infected animals (Chiers *et al.*, 2002b). The difficulty to isolate *A. pleuropneumoniae* from chronically or subclinically infected pigs has made serology a valuable tool to study the epidemiology of the microbe (Wallgren *et al.*, 1993).

1.3.1 Virulence factors

A. pleuropneumoniae has a number of different virulence factors as recently reviewed (Chiers *et al.*, 2010). The 15 serotypes of *A. pleuropneumoniae* produce different pore-forming repeats in the structural toxin (RTX) cytotoxins designated ApxI, ApxII and ApxIII which are cytolytic or hemolytic (Table 2). These toxins are secreted by the different serotypes in various combinations and determine the virulence of the different serotypes by impairing the phagocytic function of both macrophages and neutrophils (Bosse *et al.*, 2002). A fourth RTX toxin, ApxIV, is produced by all 15 serotypes but only after infection, not under *in vitro* conditions. Pigs infected with *A. pleuropneumoniae* develop specific antibodies directed against ApxIV (Dreyfus *et al.*, 2004).

Table 2. *The Apx toxins produced by the different serotype of A. pleuropneumoniae and the pathogenicity of these serotypes.*

Apx toxin(s)	**Virulence**	**Serotype**
I + II	High	1, 5ab, 9, 11 (10, 14)
II + III or II	Moderate	2, 4, 6, 7, 8, 12, 15 (13)
III	Low	3

Other factors which also determine virulence are capsular polysaccharides (CPS), lipopolysaccharides (LPS) and outer membrane proteins (OMP) (Bandara *et al.*, 2003; Dubreuil *et al.*, 2000). CPS which determines serotype specificity protects the bacterium from phagocytosis and complement-mediated killing. Variations in composition, structure and amount of CPS account for variations in virulence as non-encapsulated variants and bacteria with thin layers of CPS are less virulent (Bandara *et al.*, 2003; Dubreuil *et al.*, 2000). On the other hand, similarities in CPS together with the type of toxins produced, account for the cross-reactivity observed between different serotypes.

LPS are essential structural components found in the cell membrane of Gram-negative bacteria. Most LPS are composed of three regions; the lipid A, the core oligosaccharide and the O-polysaccharide and are denoted as smooth. Strains that have lost the O-polysaccharide are classified as rough (Dubreuil *et al.*, 2000). An intermediate form, semi-rough, also exists. The composition and structure of the LPS O-side chains are specific for most serotypes. The virulence exerted by LPS is through its ability to induce the production of pro-inflammatory cytokines (Ramjeet *et al.*, 2005) and its adhesive properties allowing for *A. pleuropneumoniae* to adhere to porcine respiratory tract cells and mucus. Mutations in LPS have been associated with a reduced capability to adhere.

OMP profiles differ between serotypes of *A. pleuropneumoniae* but there are some OMPs which are present in almost all serotypes (Ramjeet *et al.*, 2008). Conserved OMPs between serotypes include transferring-binding protein (TfbA), a 42-kDa protein, the 14-kDa peptidoglycan-associated lipoprotein (PalA) and the 50-kDa lipoprotein OmlA. OMPs play a role in iron-acquisition and since iron is essential for the growth of *A. pleuropneumoniae,* there are several iron-acquisition mechanisms designed to obtain iron from the host. Extracellular iron in the host is bound to the glycoproteins lactoferrin and transferrin while intracellular iron is mainly found in hemoglobin (Jacques, 2004).

Other virulence factors include proteases, ureases, fimbriae and superoxide dismutase. Proteases can degrade IgA and hemoglobin when released from *A. pleuropneumoniae.* Urease which may increase intracellular survival and impair macrophage function, is produced by most strains of *A. pleuropneumoniae* (Chiers *et al.*, 2010). Fimbriae are involved in the attachment of bacteria to epithelial cells of the respiratory system (Bosse *et al.*, 2002).

1.3.2 Pathogenesis and immunology

Although *A. pleuropneumoniae* can be detected in tonsils of infected pigs (Vigre *et al.*, 2002; Chiers *et al.*, 1999), its main site of action is in the lower parts of the respiratory tract which it colonizes after inhalation. Here, adhesion to mucus, proteins, ciliated cells of bronchioli and alveolar epithelial cells occurs. Adhesion involves virulence factors such as fimbriae, LPS and OMP (Chiers *et al.*, 2010). In the lower respiratory tract, *A. pleuropneumoniae* causes extensive tissue damage, often being fatal. The tissue

damage is caused by a number of factors. As nutrients are scarce in the lower respiratory tract, *A. pleuropneumoniae* has developed a number of mechanisms for obtaining nutrients from its host that also determine the virulence of *A. pleuropneumoniae*. Secreted LPS and RTX toxins induce lysis of alveolar epithelial cells, endothelial cells, red blood cells, neutrophils and macrophages, whereby necessary nutrients are released to the environment, but also resulting in tissue damage (Chiers *et al.*, 2010).

The immune system of the pig is crucial in the defence against invading pathogens. The first line of defence against infections with *A. pleuropneumoniae* is the mucosa of the respiratory tract which traps and then removes invading bacteria. However, when bacteria reach the lower respiratory tract, they adhere to epithelial cells by LPS (Auger *et al.*, 2009). LPS and RTX toxins induce lysis of alveolar epithelial cells, endothelial cells, red blood cells, neutrophils and macrophages, resulting in extensive tissue damage (Chiers *et al.*, 2010). Further, the RTX toxins produced by *A. pleuropneumoniae* will impair the phagoycytic function of the pulmonary intravascular macrophages (Frey, 2003; Haesebrouck *et al.,* 1997).

The tissue damage that arises from the released toxins results in an increased accumulation of neutrophils in lung tissue (Abraham *et al.*, 2000) and the activation of macrophages and dendritic cells. These cells respond by synthesizing and secreting cytokines and other pro-inflammatory molecules that trigger inflammation. Important cytokines include interleukin 1 (IL-1) (Baarsch *et al.*, 2000), tumor necrosis factor α (TNF-α) (Huang *et al.*, 1999), IL-6 (Johansson *et al.*, 2001; Fossum *et al.*, 1998) and Il-8 (Bosse *et al.*, 2002; Baarsch *et al.*, 2000). In turn, the inflammatory lipids prostaglandins and leukotrienes are released, leading to fever and the synthesis of acute-phase proteins in the liver (Petersen *et al.*, 2004). TNF-α triggers the local release of chemokines and cytokines which in turn promotes the adherence, migration and activation of leukocytes, primarily neutrophils at the site of tissue damage (Huang *et al.*, 1999). A transcriptional analyse of lung tissue revealed that IL-1α, IL-1β, IL-6 and IL-8 were upregulated in lungs infected with *A. pleuropneumoniae* serotype 5B whereas IL-10 and IFN-γ were downregulated. In addition, mRNA for a number of chemokines as well as for complement components and acute-phase proteins were clearly upregulated (Mortensen *et al.*, 2009).

Acute-phase proteins

Acute-phase proteins are mainly synthesized in the liver in response to inflammation, infection and tissue damage (Murata *et al.*, 2004; Petersen *et al.*, 2004) although local production in other organs such as the lung has been described (Phatsara *et al.*, 2007). Acute-phase proteins have been studied aiming to find an objective tool for measuring the influence of rearing conditions (Amory *et al.*, 2007), monitoring the progression of infections (Sorensen *et al.*, 2006; Knura-Deszczk *et al.*, 2002; Heegaard *et al.*, 1998), efficacies of different treatments (Hulten *et al.*, 2003; Lauritzen *et al.*, 2003) and the general health status of pig herds (Petersen *et al.*, 2002).

Important acute-phase proteins in the pig include serum amyloid A (SAA), haptoglobin, Pig major acute-phase protein (Pig-MAP) and C-reactive protein (CRP). The acute-phase proteins both act as stimulators and suppressors of inflammation. The different acute-phase proteins have different functions where haptoglobin has a bacteriostatic effect by binding to hemoglobin in order to prevent the loss of iron (Petersen *et al.*, 2004). Serum amyloid A (SAA) is believed to possess various inhibitory functions of the inflammatory response (Petersen *et al.*, 2004) and mannan-binding lectin (MBL) plays a role in the innate immune response by binding to bacteria (Lillie *et al.*, 2006) and activating the complement pathway (Juul-Madsen *et al.*, 2006). Pig-MAP is an acute-phase protein expressed in response to acute inflammation in pigs (Gonzalez-Ramon *et al.*, 1995; Lampreave *et al.*, 1994).

Antibodies

One important role of antibodies is to neutralize toxins and impair attachment of bacteria to epithelial cells. Humoral immunity with antibody production is considered to be of major importance in the defence against *A. pleuropneumoniae*-infections, both locally and systemically (Krejci *et al.*, 2005; Nechvatalova *et al.*, 2005; Cruijsen *et al.*, 1995b). Antibodies are mainly secreted from plasma cells situated in regional lymph nodes and in the submucosa of the respiratory tract. The local antibody response following stimulation with *A. pleuropneumoniae*-antigen can be detected in bronchoalveolar lavage fluid (BALF) (Faldyna *et al.*, 2005; Krejci *et al.*, 2005; Nechvatalova *et al.*, 2005; Hensel *et al.*, 1995) and the systemic response in serum. As *A. pleuropneumoniae* is toxigenic, not only the bacteria but also the toxins need to be neutralized (Cruijsen *et al.*, 1995b). Toxin neutralization occurs when antibodies prevent toxin from binding to receptors on target cells (macrophages).

There are five isotypes of porcine immunoglobulins; IgG, IgM, IgA, IgE and Ig D with six subisotypes of porcine IgG and two subisotypes of IgA (Butler *et al.*, 2009). IgG is the predominant immunoglobulin in serum (35 mg/mL), constituting more than 80% of the total amount of the serum Igs in pigs (Roth, 2006). IgG is important in the systemic defence against pathogens. It is the smallest immunoglobulin composed of two identical light chains and two heavy chains. It can easily leave blood vessels and enter inflamed tissues where it binds to antigens of *A. pleuropneumoniae* causing agglutination and opsonisation (Crawley & Wilkie, 2003). IgG is most abundant in the upper parts of the respiratory tract (Wilkie, 1982).

IgA is the major immunoglobulin of mucosal surfaces (53.6 μg/mL in BALF) and only exists at low levels in serum (1.3 mg/mL)(Butler *et al.*, 2009). It is secreted as a dimer and is essential in the protection of the respiratory tract. IgA cannot activate the classical complement pathway, nor can it act as an opsonin. It may however agglutinate antigens thus preventing the adherence of pathogens to cells of the respiratory tract (Roth, 2006). IgA predominates in the upper part of the respiratory tract (Wilkie, 1982).

IgM exists as a pentamer and is the second most common immunoglobulin in serum (6.5 mg/mL)(Butler *et al.*, 2009). IgM is the major immunoglobulin produced during a primary immune response. Due to their large size, IgM rarely enters sites of local inflammation (Roth, 2006).

IgE is like IgA produce locally by plasma cells located beneath body surfaces. IgE trigger inflammation by binding to receptors on mast cells and basophils which in turn release inflammatory mediators. IgE confers immunity to parasites and is involved in Type I hypersensitivity reactions (Roth, 2006). The role of IgD in pigs yet remains unclear (Butler *et al.*, 2009).

Antibodies act in different ways to remove bacteria from the airways and inactivate toxins. By binding to antigenic structures on the surface of *A. pleuropneumoniae* antibodies act as opsonins facilitating phagocytosis of the bacteria. Antibodies directed to bacterial toxins, bind toxin and neutralize them (Cruijsen *et al.*, 1995b). *A. pleuropneumoniae* has on the other hand developed mechanisms to evade antibody mediated defences. It secretes proteases which degrade IgG and IgA but it is not known if these proteases impair adhesion, opsonisation or toxins (Chiers *et al.*, 2010). Although *A. pleuropneumoniae* is opsonised by antibodies specific for CPS, complement-mediated killing is not induced and *A. pleuropneumoniae* is protected by the

CPS and LPS from phagocytosis and complement-mediated killing (Chiers *et al.*, 2010; Bandara *et al.*, 2003; Bosse *et al.*, 2002).

Once a pig has developed an acquired immunity towards *A. pleuropneumoniae,* it will respond more rapidly and efficiently compared to the first encounter with a particular microbe (Krejci *et al.*, 2005).

Fetal and Maternal immunity

The placenta of the sow is of the epitheliochorial type which prevents passage of immunoglobulins. The pig fetuses should develop in a sterile environment in the uterus, but they are capable of mounting an immune response from around 55 to 70 days of gestation (Sinkora & Butler, 2009; Tlaskalova-Hogenova *et al.*, 1994). Thus, piglets are capable of mounting an immune response at birth when they will encounter a multitude of different microorganisms. However, this capacity is limited and takes time. Therefore, piglets are dependant on receiving antibodies via colostrum directly after birth for survival.

Sow colostrum contains 21.2 mg/mL IgA, 9.1 mg/mL IgM and 95.0 mg/mL IgG (Butler *et al.*, 2009). As the levels of antibodies obtained by piglets vary depending on the immune status of the sow, this may imply that piglets derived from sows with higher level of antibodies may be better protected since they will receive higher amounts of antibodies via colostrum (Damm *et al.*, 2002; Wallgren *et al.*, 1998). The transfer of preformed antibodies from immune sows to naïve piglets will confer an immediate but temporary immunity in the piglets. This immunity will persist until the passively derived antibodies have been catabolised.

1.3.3 Epidemiology and clinical signs

The first report of pneumonia in pigs associated with *A. pleuropneumoniae,* or *Haemophilus parainfluenzae* as it was named then, was made in 1957 (Pattison *et al.*). In 1961, a severe outbreak of respiratory disease in Argentinean pigs was associated with *A. pleuropneumoniae* was described (Shope, 1964). The disease symptoms described agree well with those observed in acute outbreaks of today.

Clinical signs include per acute deaths without recording of any preceding symptoms, most often observed during the fattening period. In acute cases, forced breathing with an increase in frequency, sitting posture and fever of more than 40° C are common signs. If pigs with severe signs are left

untreated, death usually follows within the next few days. Less severely affected animals may recover and the course of disease becomes chronic. In the chronic stage of disease, pigs may cough intermittently; appetite may be reduced and there is little or no fever (Gottschalk 2006). The severity of the disease depends on the age when pigs become infected (Sebunya *et al.*, 1983), their state of immunity (Krejci *et al.*, 2005; Nechvatalova, 2005; Cruijsen *et al.*, 1995b), the environment (Beskow, 2008; Beskow *et al.*, 1998), season (Maes *et al.*, 2001), infectious dose (Velthuis *et al.*, 2002; Vigre *et al.*, 2002; van Leengoed & Kamp, 1989; Sebunya *et al.*, 1983) and which serotype the pigs encounter (Rosendal *et al.*, 1985).

Herds become infected by either the purchase of carrier animals or by poor biosecurity measures (Maes *et al.*, 2001). Once an infection becomes established, further transmission of the infection mainly occurs by pig-to-pig contact (Velthuis *et al.*, 2002; Jobert *et al.*, 2000; Savoye *et al.*, 2000). The infection is generally associated with high morbidity and varying mortality rates when first introduced, but as immunity on herd level develops, the course of disease becomes more chronic in character. The animals which were only subjected to low doses or survived the initial infection become subclinical carriers and will make the infection endemic on herd level (Velthuis *et al.*, 2002).

Apart from direct transmission, there are reports on transmission over short distances by aerosol under experimental conditions (Kristensen *et al.*, 2004; Jobert *et al.*, 2000). It has also been demonstrated that within herd transmission occurs between separate units in large herds housing several age categories within the same building despite employing all-in all-out management within each unit of the building (Nielsen, 2000).

Another important transmission route is from sow to offspring, and piglets may harbour *A. pleuropneumoniae* in their tonsils already at 11 days of age (Vigre *et al.*, 2002). Although disease rarely occurs at this age due to the presence of maternal antibodies (Cruijsen *et al.*, 1995a; Cruijsen *et al.*, 1995b; Nielsen, 1995), the piglets may transmit the infection to non-immune pigs later on during production when maternal immunity wanes (Chiers *et al.*, 2002a; Vigre *et al.*, 2002). This is reflected by an increase in the cumulative proportion of pigs harbouring *A. pleuropneumoniae* in their tonsils between the age of four to 12 weeks (Vigre *et al.*, 2002).

Production systems also greatly influence transmission and the outcome of *A. pleuropneumoniae* infections. All-in all-out production systems have proven very effective in reducing the impact of respiratory disease caused by *A. pleuropneumoniae* while continuous systems predispose for outbreaks of respiratory disease (Cleveland-Nielsen *et al.*, 2002; Ice *et al.*, 1999). Disease manifestations also vary over time within production systems which could be a seasonal effect or reflect the level of herd immunity (Maes *et al.*, 2001; Beskow *et al.*, 1998) Also, the purchase of pigs increase the risk for respiratory disease, especially if grower pig are bought from multiple sources by fattening herds (Rosendal & Mitchell, 1983).

1.3.4 Diagnosis

Apart from bacterial cultivation which is considered the "gold standard", there are other ways of diagnosing infections with *A. pleuropneumoniae* both on an individual basis and on herd level (Stark, 2000). This is of importance since cultivation is difficult in cases other than acute ones in which the bacterium can be isolated from affected lung tissue and possibly in BALF (Moorkamp *et al.*, 2008; Palzer *et al.*, 2008)

Serology is commonly used to detect antibodies to *A. pleuropneumoniae*. Antibodies are usually not detectable until two weeks post infection, although antibodies to *A. pleuropneumoniae* may appear already seven days post infection (Wallgren *et al.*, 1999a). There are a number of serological tests, but cross-reactions between serotypes may limit their usefulness (Table 3). Antibodies may also be detected in milk samples from sows and from muscle fluids, but the antibody concentrations are generally lower than in blood (Wallgren & Persson, 2000; Levonen *et al.*, 1994).

Table 3. *Cross-reactivity between different serotypes of Actinobacillus pleuropneumoniae and the isolation of these serotypes in Sweden.*

	***Actinobacillus pleuropneumoniae* serotype**														
	1	**2**	**3**	**4**	**5a, 5b**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
Cross-reactions	**9, 11**	**-**	**6, 8**	**7**	**-**	**3, 8**	**4**	**3, 6**	**1, 11**	**-**	**1, 9**	**15**	**-**	**-**	**12**
Found in Sweden	**-**	**X**	**X**	**X**	**X**	**X**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**

The PCR technique combines high sensitivity with high specificity making it a valuable tool in diagnosing *A. pleuropneumoniae* infections. Asymptomatic carriers of *A. pleuropneumoniae* can be identified by analyzing samples

collected from tonsils (MacInnes *et al.*, 2008; Chiers *et al.*, 2002b). The PCR technique is also capable of rapid serotyping of *A. pleuropneumoniae* strains (Stark, 2000). However, there are limitations with PCR, as new variants of *A. pleuropneumoniae* may not be detected. This limitation is also true for antibody-detecting methods, observed when atypical strains of serotype 12 (Blackall *et al.*, 2002) and serotype 5 (Wallgren *et al.*, 2003) infected pigs in Australia and Sweden, respectively

1.3.5 Prevention and control

Infections with *A. pleuropneumoniae* can be successfully treated using a range of different antimicrobials provided that the isolates are susceptible (Herradora & Martinez-Gamba, 2003; Luque *et al.*, 2000; Wallgren *et al.*, 1999b; Wallgren *et al.*, 1999a; Smith *et al.*, 1991). However, there is an increasing number of isolates which are resistant to several antimicrobials which may pose a problem in herd health management (Matter *et al.*, 2007; Gutierrez-Martin *et al.*, 2006; Chang *et al.*, 2002). Other important methods that can reduce the impact of *A. pleuropneumoniae* infections are cleaning and disinfection (Beskow *et al.*, 2008; Beskow *et al.*, 1989).

Passive immunity may play a role in disease prevention of young pigs and there is a potential for reducing transmission of *A. pleuropneumoniae* within endemically infected herds (Krejci *et al.*, 2005; Nechvatalova *et al.*, 2005).

In order to control infections with *A. pleuropneumoniae*, different preventive measures have been employed. Depopulation and repopulation with SPF-animals is a safe method provided that correct biosecurity measures are undertaken following repopulation. Attempts to eradicate *A. pleuropneumoniae* in existing herds have been employed with varying degrees of success (Stark *et al.*, 2007; Hofmo & Lium, 1998; Larsen *et al.*, 1998). Therefore, the employment of age-segregated rearing which has been proven successful in reducing the impact of both acute and subclinical disease (Tarasiuk & Bzdawka, 2010; Cleveland-Nielsen *et al.*, 2002; Ice *et al.*, 1999), probably is the most commonly used measure to control actinobacillosis.

1.3.6 Control by vaccination

Vaccination can be an efficient method of controlling disease which was demonstrated already in 1798 by Edward Jenner when material from cowpox lesions were used to protect humans from smallpox and later the process was refined by Louis Pasteur (Silvers & Steptoe, 2001). In general, vaccination is used to induce immunity towards defined pathogens in naïve

individuals, providing protection against disease. Vaccinations against actinobacillosis have also been employed (Ramjeet *et al.*, 2008; Haesebrouck *et al.*, 2004; Valks *et al.*, 1996; Beskow *et al.*, 1993; Beskow *et al.*, 1989), but have generally not been very effective unless other preventive measures have also been undertaken.

In order to achieve optimal protection, an antigenic component of the pathogen of interest must be delivered in a fashion so that immunity will be induced at the site of infection. Therefore, knowledge of host-pathogen interactions is crucial in vaccine development and vaccination of pigs. To date, a number of different antigenic components have been used in vaccines against *A. pleuropneumoniae* infections but so far without complete satisfaction (Ramjeet *et al.*, 2008; Haesebrouck *et al.*, 2004). The first vaccines consisted of heat-killed bacteria or formalin-treated whole cells having the advantage of presenting a multitude of antigenic determinants. These vaccines were administered intramuscularly since killed bacteria are unable to colonize the respiratory tract which is a prerequisite for local immunity to develop. Being a respiratory pathogen, local immunity is considered to be important in the defence against *A. pleuropneumoniae* infections (Nechvatalova *et al.*, 2005) which may explain the poor efficacy of intramuscularly administered bacterins (Ramjeet *et al.*, 2008; Haesebrouck *et al.*, 2004).

As lesions to a large extent are caused by the different toxins of *A. pleuropneumoniae*, the Apx toxins together with OMP are considered to be major antigens and have consequently been in focus when designing subunit vaccines (Ramjeet *et al.*, 2008; Haesebrouck *et al.*, 2004). However, these vaccines did not either confer complete protection (Oldfield *et al.*, 2008; Van Overbeke *et al.*, 2001). Recent development using live attenuated *A. pleuropneumoniae*-vaccines has showed promising results but are not yet commercially available (Maas *et al.*, 2006).

2 Aims

The overall objective of this thesis was to investigate respiratory infections in pigs, especially those caused by *Actinobacillus pleuropneumoniae,* both under field and experimental conditions.

More specifically the aims were to:

- Increase the knowledge on passive immunity to infections with *A. pleuropneumoniae* and how variations in the passive immunity provided by the sow via colostrum may influence piglet susceptibility to infections and the overall performance of these pigs from birth to slaughter.
- Study the course of *A. pleuropneumoniae*-infections in relation to the protective role of innate and acquired immunity at repeated exposures to the pathogen.
- Obtain further knowledge on how secondary infections with *Pasteurella multocida* may influence the epidemiology of *A. pleuropneumoniae*-infections on herd level.
- Evaluate potential strategies in preventing and combating infections with *A. pleuropneumoniae*.

3 Considerations regarding Materials and Methods

Considerations regarding materials and methods used in this thesis are presented below. Detailed information is given in each paper (I-IV).

3.1 Field trials

The studies described in papers I and IV were conducted as field trials. The unique conditions of every herd will of course influence the results. This will in turn certainly limit to what extent the results can be applied to other herds.

Factors which can have an impact on respiratory infections are: herd type (specialized piglet producing herd, fattening herd, farrow-to-finish herd), production system (continuous or all-in all-out), ventilation system, air volume, feeding regimes including feed components, recruitment and quarantine policies (Beskow *et al.*, 2008; Cleveland-Nielsen *et al.*, 2002; Maes *et al.*, 2001; Ice *et al.*, 1999; Beskow *et al.*, 1998). Also seasonal variations are known to play a role in *A. pleuropneumoniae*-infections (Maes *et al.*, 2000). These factors may also change over time. Therefore, the results obtained in field trials should be applied with care to other herds. Still, field trials are required to test hypothesis and the effect of remedial measures under real life conditions.

3.2 Experimental trials

Experimental trials allow for controlled conditions surrounding the factors investigated. Thus, the results obtained are more likely to truly reflect the parameters investigated (Papers II and III). However, when the same parameters are investigated under field conditions, the outcome may be different since a number of other variables are likely to interact with the investigated variable thereby influencing the results.

Experimental trials are often very costly due to rigorous biosecurity demands which also put limitations on the size of research facilities suitable for experimental trials. This will limit the numbers of animals that can be used in a trial, especially if several trial groups are used which the case for the experimental trial was, described in papers II and III. A small number of animals can result in great standard deviations in which true significant differences may be obscured by the out-layers.

Figure 2. View of the research facilities at SVA. This group of pigs had been treated with tetracycline after an inoculation with *Actinobacillus pleuropneumoniae* and was later reinoculated with the same strain (Papers II and III).

3.3 Selection of herds and animals.

3.3.1 Paper I

For the study described in paper I, a farrow-to-finish herd suffering from an endemic infection with *A. pleuropneumoniae* was selected. The herd investigated weaned their piglets at the age of six and a half weeks which was later than the average weaning age (33.9 days) in Sweden. After weaning, piglets were kept in their pen of birth until the age of nine weeks. More commonly piglets are either transferred to a growing unit at weaning, or kept in the combined farrowing-growing pens until they are either sold or transferred to the fattening unit.

The production was age segregated until the age of nine weeks, but after that it was continuous although pigs were housed batch-wise. Pigs of different ages were not mixed in the pens. At the age of 16 weeks, the growers were transferred to the fattening unit housing five different age categories. Neither in the fattening unit were pigs of different ages mixed on pen level. The continuous production system contributed to the endemic disease situation as it has been shown that all-in – all-out production has greatly reduced the impact of respiratory infections (Cleveland-Nielsen *et al.*, 2002; Ice *et al.*, 1999).

The sows of paper I were selected with the aim of obtaining dams which differed in serum antibody concentrations to *A. pleuropneumoniae* within the same batch of sows. Pregnant gilts were excluded in order to avoid animals with an active, ongoing infection. As runts are more likely to die before weaning, these animals were excluded with the aim minimizing loss of animals during the trial.

3.3.2 Papers II & III

The selection of the pigs used for the experimental trial was based on the health status and age of the pigs. As it was necessary to use pigs previously un-exposed to *A. pleuropneumoniae* in order to be able to study the primary immune response, SPF pigs were required. The pigs were nine weeks old when arriving at the research facilities and 10 weeks when they were inoculated in order to correspond to the age when pigs in conventional herds are usually allocated to fattening herds/units and thereby risk being subjected to infections with *A. pleuropneumoniae* (Andreasen *et al.*, 2000; Wallgren *et al.*, 1993).

3.3.3 Paper IV

The herd selected for the study described in paper IV, was a good representative of a modern specialized fattening herd in Sweden having good production records and being well-managed. As it had experienced an acute outbreak of actinobacillosis recently, it was possible to test preventive measures in the form of vaccinations in a controlled manner under field conditions.

The herd was monitored for two years, and during this time the suppliers of growers changed. As infectious pressures vary between herds, this may have influenced the epidemiology in the fattening herd. During the time period when pigs were vaccinated twice, all sampled animals were seronegative on arrival while six sampled pigs were seropositive already on arrival when pigs were vaccinated three times. The suppliers of growers were different during these two periods which most likely influenced the results. This on the other hand, reflects pig production as it organized in Sweden today.

3.4 Isolate used for inoculation

A. pleuropneumoniae serotype 2, strain NVI 700/89, was used for the experimental study described in papers II and III. Serotype 2 is the predominant serotype found in Sweden and the isolate used was cultivated in 1989 from a clinical case of acute actinobacillosis. The strain belongs to a clone which is still dominating in Sweden as confirmed by pulsed-field gel electrophoresis (PFGE) (Aspàn & Wallgren, 2008). This particular strain has previously been used in other experimental studies (Wallgren *et al.*, 1999b; Wallgren *et al.*, 1999a). An inoculation dose which would induce acute actinobacillosis without killing the pigs could therefore be used. Since the strain belonged to the predominant serotype in Sweden, it did add strength to the results although these were obtained from an experimental study.

3.5 Diagnostic methods

3.5.1 Enzyme-linked immunoassay (ELISA)

Antibody levels to *A. pleuropneumoniae* serotype 2 were detected by an indirect ELISA system detecting the total amount of Ig's (Wallgren & Persson, 2000). In brief, the ELISA is based on a phenol-water extract of an *A. pleuropneumoniae* serotype 2 isolate which is used as the coating antigen. The antigen contains no proteins. The cut-off value for a positive reaction

in sera diluted 1/1000 in PBS-T was defined as A_{450}=0.50. When a cut-off value of 0.50 (A_{450}) is used, the ELISA has a high specificity (99%) and high sensitivity (97%) (Wallgren & Persson, 2000). This implies that also low responding pigs will be detected with this ELISA.

The ELISA used detects LPS and CPS antigens which pigs produce large amounts of antibodies against. Although LPS antigens are considered to be serotype-specific, there are antigenic similarities which cause cross-reactivity in serological assays (Dubreuil *et al.*, 2000) (Table 3).

3.5.2 Acute-phase proteins

SAA and haptoglobin were analysed using commercial assays. These assays have been validated in intra and inter assay tests (Tecles *et al.*, 2007). The coefficients of variation were low for the haptoglobin assays when samples obtained from both SPF and diseased pigs were analysed. In contrast, there was a large coefficient of variation for the SAA assays. It would therefore have been worthwhile to have analysed the samples from the experimental trial (Paper III) in duplicates. However, a clear response was detected for both of these acute-phase proteins in the experimental trial described in paper III.

According to the manufacturer, haptoglobin levels up to 2.2 mg/mL are considered to be within the normal range and samples which are classified as acute are within the range of 3.0 to 8.0 mg/mL. The highest level of haptoglobin obtained in the trial (Paper III) was from the most severely diseased pig but it did only reach 2.02 mg/mL. Still, there was a clear difference in serum levels of haptoglobin between diseased and non-diseased pigs which makes the test results valid although they were of a lower magnitude. The highest SAA concentrations in the experimental trial were on the other hand greater than the reference range provided by the manufacturer (62.5 – 1000 µg/mL). Although the observed differences between the results obtained in the experimental trial and the concentrations suggested by the manufacturer, this test was considered to be useful in assessing *A. pleuropneumoniae* infections in SPF pigs as there were definite increases in SAA following the inoculations of naïve animals.

Pig-MAP was analysed according to a previously described protocol (Gonzalez-Ramon *et al.*, 1995; Lampreave *et al.*, 1994) and not with the commercially available test that has been validated (Tecles *et al.*, 2007). The concentrations obtained in SPF pigs in the experimental trial (Paper III)

were lower compared to concentrations obtained from eight-week old pigs in commercial Spanish herds (Pineiro *et al.*, 2009). Most likely this difference mirrors the difference in health status between these two groups of pigs.

Porcine MBL described in paper III, is the most recently identified of the acute-phase proteins investigated and there is no commercial assay available for the analysis of pMBL. The samples analysed in paper III exhibited a large variation in serum concentrations of pMBL. This difference was not considered to be due to variations in the assays as similar variations have been described previously and attributed to differences in heritability between breeds (Juul-Madsen *et al.*, 2006). The divergent samples were re-analysed one or two more times but continued to have deviating values. Thus the low values obtained for these samples were considered to be true.

3.6 Antimicrobials

The selection of antimicrobials used in the experimental trial described in papers II and III, was based on prior knowledge of treatment efficacies of antimicrobials against *A. pleuropneumoniae*-infections (Lauritzen *et al.*, 2005; Herradora & Martinez-Gamba, 2003; Lauritzen *et al.*, 2003; Luque *et al.*, 2000; Wallgren *et al.*, 1999b; Wallgren *et al.*, 1999a; Smith *et al.*, 1991).

Enrofloxacin was selected for its reported superior efficacy in treating infections with *A. pleuropneumoniae* (Herradora & Martinez-Gamba, 2003; Wallgren *et al.*, 1999b; Wallgren *et al.*, 1999a). Tetracyclines have also been used as in-feed medications with reported good results (Luque *et al.*, 2000). Penicillin has been used to treat actinobacillosis in clinical practice with reportedly good results[1], but has in experimental trials been less efficacious (Wallgren *et al.,* 1999a). There are no reports of clinical trials where penicillin is used for treating *A. pleuropneumoniae*-infections but, amoxycillin has been reported efficacious in treating actinobacillosis (Lauritzen *et al.*, 2005).

The minimal inhibitory concentration (MIC) using broth microdilution according to the recommendations of the National committee for clinical laboratory standards (NCCLS) was determined for the isolate (*A. pleuropneumoniae* serotype 2 NVI strain 700/89) used for inoculation

[1] Magnus Paulsson, personal communication

(Wallgren *et al.*, 1999b; Wallgren *et al.*, 1999a). The MIC values were determined to 0.5 μg/mL for penicillin, 0.25 μg/mL for enrofloxacin and 1.0 μg/mL for oxytetracycline (Wallgren *et al.*, 1999a). With these MIC values, the isolate used was considered sensitive to the antimicrobials used for treatment.

In this way, differences observed for the parameters studied could be directly linked to the pharmacological properties of the antimicrobial used for treatment. A broad range of efficacies was chosen in order to investigate how this affected the immune responses.

3.7 Vaccine & vaccination time points

The vaccine, Porcilis® APP vet. (Intervet, Boxmeer, the Netherlands), used in the study described in paper IV, is the only commercial vaccine against *A. pleuropneumoniae* currently available in Sweden (FASS vet., 2010). It is a subunit vaccine which contains OMP proteins, the Apx toxoids; Apx I, Apx II and Apx III, together with dl-α-tochopherolacetate as adjuvant. These antigens are considered to be major virulence factors of *A. pleuropneumoniae* and have therefore been considered as important components in vaccines (Ramjeet *et al.*, 2008). However, the vaccine has been used rather sparsely, possibly due to its reported limited efficacy (Ramjeet *et al.*, 2008; Haesebrouck *et al.*, 2004; Chiers *et al.*, 1998).

Reduced vaccine efficacy could have a number of explanations, some of which may be due to incorrect handling including incorrect administration and/or incorrect storage temperature. As it was possible to control for, these parameters were ruled out as causes of reduced vaccine efficacy.

The time-point for administration could be discussed. In the trial described in paper IV, pigs were vaccinated on arrival at the fattening herd. The age when pigs are brought to a fattening herd varies but ranges from eight to 14 weeks. According to the product details of the vaccine, the vaccine should be administered at the age of six and 10 weeks to ensure that pigs are vaccinated before they are likely to become infected. However, when pigs are bought from different piglet producers, it is not always known in advance which herds these pigs originate from. This makes it difficult to vaccinate pigs at the stipulated ages unless you raise your own pigs. Vaccinations against *A. pleuropneumoniae* are therefore usually performed when pigs arrive at the fattening herds.

Another limitation is that the efficacy of the vaccine is reported to be of short duration (FASS vet., 2010). The serological response measured after vaccination was greatest two weeks post vaccination and declined thereafter (Ridremont *et al.*, 2006). This implies that antibody level will be greatest at the time-point of arrival to a fattening herd if pigs are vaccinated at the age of six and 10 weeks. Further, the antibody levels will decline during the fattening period indicating a reduced protection during the later stages of rearing. Depending on the infection dynamics of the herd, pigs may be unprotected to *A. pleuropneumoniae* infections although they have been vaccinated (Andreasen *et al.*, 2000).

Another aspect on the study described in paper IV, was the decision to vaccinate entire batches under a period of time and then completely cease with the vaccinations for a period of time. In order to be able to compare the effect of vaccinations on performance it would have been desirable to vaccinate half the batch and leave the other half as a control. However, as respiratory pathogens may spread between units in large herds housing several age categories within the same building despite all-in all-out management (Nielsen *et al.*, 2000), it was decided that all pigs under a defined time period would be vaccinated and that adjacent time periods when no vaccinations were employed would serve as control periods.

3.8 Slaughter registrations

Registrations made in the post mortem inspection of carcasses at slaughter provide valuable information on the lung health status of a herd. However, pleuritis registrations do not provide information on the etiologic cause of the lesions (Jirawattanapong *et al.*, 2009). Further, if a lung pluck is registered for pleuritis, there is no indication on the extent of the lesions. All lesions larger than two centimetres in diameter will be classified as pleuritis. This means that a pleurisy lesion covering most of the lung lobes will have the same dignity as the smallest lesion recorded in slaughter registrations. The slaughter registrations will therefore provide little information on the severity of disease on herd level. Thus, a herd registered for 30% pleuritis will not necessarily be more severely affected than a herd registered for *e. g.* 15% pleuritis. However, slaughter registrations together with production parameters such as daily weight gain (DWG) will be useful in assessing respiratory disease on herd level.

3.9 Statistical analyses

As the majority of data and variables analyzed were non-normally distributed, the Wilcoxon Rank Sum test was used in the statistical analyses of continuous data (Papers I, III and IV). Differences between experimental groups in the whole blood culture stimulatory response described in paper III were analyzed for each sampling day using the Mann-Whitney test. Non-continuous data were categorized and analyzed using the Fischer's Exact test (Paper IV). Area-under-curve (AUC) calculations for blood parameters for every individual over three-day periods, except the last period which contained four days, were used when groups were compared as described in paper III.

In Paper II, *t*-test was used for comparison between recordings made at group level or once per pig. An analysis of variance (PROC MIXED) was applied to variables that were recorded for individual pigs' data. The statistical model included fixed effects of group, sex, time period and sampling day within time period, and the interaction between group and sex and between group and time period. The statistical model also included the random effect of pig, nested within group/sex combinations. Least square means were estimated and compared using a *t*-test.

4 Main Results and Discussion

The resistance of individual pigs to infections with *A. pleuropneumoniae* depends on a number of variables and starts even before they are born. Serum concentrations of antibodies to *A. pleuropneumoniae* serotype 2 were found to decrease during the last month of gestation among sows with high serum antibody concentrations (Paper I) which was in accordance with previous findings (Klobasa *et al.*, 1985). Antibodies are transferred from serum to the mammary gland (Salmon *et al.*, 2009), and higher levels of colostral antibodies were detected in colostrum of sows with high levels of serum antibodies than in colostrum of sows with low levels of serum antibodies (Paper I), which has also been shown for other microbes (Damm *et al.*, 2002; Wallgren *et al.*, 1998). Thus, the levels of antibodies in colostrum appear to be related to the levels of serum antibodies, which indicate that the concentration of colostral antibodies can be enhanced through prior stimulation of systemic immunity of the sows. This implies that there are ways to improve immunity in neonatal pigs.

Although actinobacillosis is usually seen in growers and fatteners it may be beneficial to increase the levels of serum antibodies in pregnant sows with the aim of improving the colostral transfer of antibodies to the offspring. However, this must be performed so that clinical disease is not induced. Passively derived immunity has been shown to play an essential role in the protection against disease in piglets (Krejci *et al.*, 2005; Nechvatalova *et al.*, 2005), and it has been demonstrated that higher levels of antibodies in colostrum will prolong the period in which passively derived antibodies can be detected in serum from piglets (Damm *et al.*, 2002; Wallgren *et al.*, 1998). This was now also confirmed for *A. pleuropneumoniae* (Paper I), which appears to be of clinical relevance because it has been demonstrated

that antibody levels influence the level of protection against *A. pleuropneumoniae* (Cruijsen *et al.*, 1995b).

It has been demonstrated that piglets may harbour *A. pleuropneumoniae* in their tonsils already at an age of 11 days (Vigre *et al.*, 2002) and as vaccination of young piglets against *A. pleuropneumoniae* at the age of two and five weeks did not affect the susceptibility (Velthuis *et al.*, 2003), it may be beneficial to increase the passive immunity of piglets. This could possibly reduce the number of subclinical carriers, thereby reducing transmission of the infection between young piglets.

It is however not known whether a high level of passive immunity really will reduce the number of subclinical carriers. Nor if the duration of the passively derived immunity would last long enough to be effective when pigs first encounter the pathogen, which is likely to occur at the age of 9 to 12 weeks. This age coincides with the time when maternal immunity generally has declined to low levels leaving pigs relatively unprotected to infections (Chiers *et al.*, 2002a; Cruijsen *et al.*, 1995c; Gardner *et al.*, 1991). This age also corresponds to the age when pigs from different sources are brought together in specialized fattening herds and the age when *A. pleuropneumoniae* is most readily isolated from the upper respiratory tract (Wongnarkpet *et al.*, 1999; Willson *et al.*, 1987). If unprotected pigs are mixed with subclinical carriers, there is an increased risk for disease, both among the unprotected pigs and the carriers (Velthuis *et al.*, 2002).

A common method to enhance systemic immunity is by vaccination against the pathogen of interest. It has been demonstrated that the offspring to vaccinated sows expressed higher antibody levels to *A. pleuropneumoniae* until the age of 10 weeks compared to the offspring of unvaccinated sows (Bak *et al.*, 1998). Possibly, vaccination of sows could provide piglets with enough antibodies until the age when they most likely encounter the infection. However, the offspring to the vaccinated sows were less responsive to a natural infection producing smaller amounts of antibodies compared to the offspring of unvaccinated sows (Bak *et al.*, 1998). In accordance, the offspring to the sows with high levels of antibodies to *A. pleuropneumoniae* serotype 2 seroconverted at a later time point than the offspring to sows with low antibody levels (Paper I). In contrast, they mounted higher levels of serum antibodies toward *A. pleuropneumoniae* when they seroconverted.

The influence of passively derived immunity on the levels of Ig isotypes in serum and BALF following exposure to *A. pleuropneumoniae* has been shown by others. Piglets that had received colostrum produced an IgM response in serum indicative of a primary immune response following a low-dose challenge with *A. pleuropneumoniae* at four weeks of age. However, the response was weaker compared to the response seen in piglets that had not received colostrum prior to the low-dose challenge (Nechvatalova *et al.*, 2005). The same pattern was also observed for serum IgG antibodies. In BALF, an increase in IgA antibodies was only detected in pigs which had not received colostrum when challenged. There was on the other hand an increase in BALF IgG, regardless if the piglets had received colostrum or not prior to the low-dose challenge. Still, piglets that had received both colostrum and a low-dose infection (which resemble the natural transmission dynamics) were best protected against a high-dose challenge (Krejci *et al.*, 2005; Nechvatalova *et al.*, 2005).

In paper I, the seroconversion to *A. pleuropneumoniae* was in both groups followed by increasing levels of serum antibodies to both toxigenic and non-toxigenic strains of *P. multocida*, emphasizing the important role of *P. multocida* as a secondary invader (Paper I).

The observed difference in antibody levels to *A. pleuropneumoniae* serotype 2 in sows was also reflected in their offspring, *i.e.* the offspring to sows with high levels of serum antibodies to *A. pleuropneumoniae* also developed high levels of antibodies to *A. pleuropneumoniae* (Paper I). These piglets also developed higher levels of antibodies to both toxigenic and non-toxigenic strains of *P. multocida* (Paper I). This indicates that immune traits are heritable, which has also been investigated in detail previously (Mallard *et al.*, 1998). Pigs selectively bred for high immune response were shown to be more responsive to vaccinations against *A. pleuropneumoniae* (Magnusson *et al.*, 1997). However, high immune responsiveness may be detrimental, as pigs of a high immune response line suffered from more severe clinical signs after being subjected to a *Mycoplasma hyorhinis* infection (Magnusson *et al.*, 1998).

At transfer to the fattening unit, increasing levels of serum antibodies to *A. pleuropneumoniae* were detected both in pigs with high and low initial levels of serum antibodies (Paper I). However, at this time without a subsequent decrease in weight gain, which indicated that the pigs had developed a

partial or complete protection to reinfections with *A. pleuropneumoniae* (Paper I).

To further explore this possibility, an experimental trial was designed that investigated the secondary immune response to *A. pleuropneumoniae* and how this was related to the primary immune response. The development of antibodies was associated to the protection against re-infection with *A. pleuropneumoniae* (Papers II and III).

Acute pleuropneumonia continues to be a problem in pig production in spite of preventive measures such as batch-wise rearing, biosecurity measures *et.c.* Therefore, the use of antimicrobials is required in order to reduce losses in form mortalities and reduced production performance. There are numerous studies describing the efficacies of different antimicrobials (Herradora & Martinez-Gamba, 2003; Luque *et al.*, 2000; Wallgren *et al.*, 1999b; Wallgren *et al.*, 1999a; Smith *et al.*, 1991). However, as pigs in endemically infected commercial herds are likely to be re-exposed to *A. pleuropneumoniae*, it is desirable that treated pigs also develop a protective immunity towards reinfections. Whether this is achieved or not, can depend on the efficacy of the treatment used, and on the time point when treatment is initiated.

The efficacy of enrofloxacin in treating infections with *A. pleuropneumoniae* is well documented (Herradora & Martinez-Gamba, 2003; Wallgren *et al.*, 1999b; Wallgren *et al.*, 1999a; Smith *et al.*, 1991). However, there are reports on the occurrence of widespread resistance to enrofloxacin among clinical isolates in Taiwan (Wang *et al.*, 2010), and also in a few European isolates (Hendriksen *et al.*, 2008). Therefore, it is always desirable to evaluate MIC-values when medicating with enrofloxacin against actinobacillosis.

Once again, the superior efficacy of enrofloxacin in treating acute actinobacillosis was documented (Papers II and III). However, the intiation of treatment with enrofloxacin at the very first onset of clinical signs appeared to eliminate the bacteria before a specific immune response had developed and the pigs were unprotected at reinfection. Consequently, the enrofloxacin treated pigs did not differ in clinical signs of disease from the previously uninoculated pigs following the second challenge. Acute-phase proteins are a part of the innate immune response (Murata *et al.*, 2004; Petersen *et al.*, 2004), and the acute-phase response seen at the re-exposure to *A. pleuropneumoniae* in the previously enrofloxacin treated pigs further

strengthens the observations that these pigs had not mounted a sufficient immune response following the first exposure to the microbe to be protected at he second exposure (Paper III).

The enrofloxacin treated pigs also differed from the other treated pigs in that they had not developed antibodies following the first challenge. On the other hand, the tetracycline and penicillin treated pigs as well as the untreated pigs had all developed serum antibodies to *A. pleuropneumoniae*. These pigs were unaffected by the second challenge and did not display an acute-phase response then (Papers II and III). This implied that an immune response was initiated at the first exposure to *A. pleuropneumoniae,* which was mirrored by a seroconversion to *A. pleuropneumoniae* in these pigs. However, the clinical signs in the tetracycline treated group were less prominent than in the other inoculated groups and did not differ from the enrofloxacin treated group from 44 hours after the first inoculation (Paper II).

Although the *A. pleuropneumoniae* isolate showed low MIC values for tetracycline (Wallgren *et al.*, 1999a), it is surprising that it was almost as efficient in reducing the clinical signs as enrofloxacin since tetracycline is generally considered to be bacteriostatic in its mode of action (Giguère, 2006). However, bactericidal activity against *A. pleuropneumoniae* has been demonstrated, but with a slow kill rate which may be of importance for this observation (Norcia *et al.*, 1999). Also, the anti-inflammatory properties associated with tetracycline may account for its efficacy, partly owing to the amelioration of the toxic effects of the LPS released by *A. pleuropneumoniae* (Giguère, 2006). The efficacy of the tetracycline treatment could also be seen at necropsy since these pigs had the lowest scores for both pleurisy and pneumonia lesions (Paper II). Neither could *A. pleuropneumoniae* be isolated from the sampled tissues 20 days after the second inoculation.

In contrast, *A. pleuropneumoniae* has been reisolated 17 days after a single inoculation in pigs, in-feed medicated with tetracycline initiated prior to the inoculation (Wallgren *et al.*, 1999b). It is important that correct doses are administered; otherwise there may be a lack of therapeutical effect (Hunneman, 1986). Per oral administration of antimicrobials may result in large variations in plasma concentrations between animals, possibly contributing to the reduced efficacy in preventing the development of subclinical carriers (Mason *et al.*, 2009). However, in the present study, the development of a protective immunity to *A. pleuropneumoniae* following the first inoculation was manifested as the microbe was not reisolated after the

second challenge in pigs that had been treated with tetracycline after the first inoculation with *A. pleuropneumoniae* (Papers II and III).

Although both enrofloxain and tetracycline were clinically efficacious (Paper II), only pigs treated with tetracycline did seroconvert to *A. pleuropneumoniae*. Further, they were protected from disease at the reinoculation, and did not mount an acute-phase response then (Paper III). The antibody levels to *A. pleuropneumoniae* did on the other hand increase more gradually in the tetracycline treated pigs compared to the antibody levels in the penicillin treated group and the inoculated control group. Further, the serum antibody level was lower in the tetracycline treated pigs than in the other two groups at the end of the trial, but this did not seem to influence the level of protection. This was in contrast to a previous report indicating that protection against *A. pleuropneumoniae* was dependent of the levels of antibodies (Cruijsen *et al.*, 1995b). However, it could also indicate that if a certain threshold level of antibodies to *A. pleuropneumoniae* is reached, this may be sufficient to prevent the development of disease.

Penicillin showed the least efficay in treating clinical actinobacillosis as this group did not differ from the inoculated control group in clinical recordings until day 15 following the first inoculation (Paper II). However, there was a delay in seroconversion with five days compared to the inoculated control group, indicating a partial therapeutic effect of the drug. There was no increase in clinical signs of disease following the second inoculation. Nor was an acute-phase response recorded and the leukocyte counts remained unaltered. Consequently, pigs were unaffected by the second inoculation which in turn indicated that a protective immunity had developed in these pigs, which was further supported by the inability to reisolate *A. pleuropneumoniae* at necropsy.

The previously untreated pigs resembled those treated with penicillin following the first inoculation (Paper II). The signs of clinical disease did not increase following the reinoculation with *A. pleuropneumoniae*. This indicated that the pigs had developed immunity towards the microbe, as also supported by the lack of an acute-phase response (Paper III).

The results obtained indicate that penicillin might not be the best treatment choice against *A. pleuropneumoniae* (Paper II and III). Variable responses to treatments with penicillin have also been reported from the USA (Frank *et al.*, 1992). There are possible explanations for the poor result reported for

penicillin (Paper II & III). At concentrations eight times the MIC-value, penicillin has been shown to inhibit growth of *A. pleuropneumoniae* for up to 24 hours (Norcia *et al.*, 1999). As penicillin works in a time-dependant fashion, the results may have been different if other treatment strategies had been employed. It has been suggested that penicillin needs to be administered at 12-hour intervals and at higher doses (25 instead of the 21 mg/kg bodyweight used in the experimental trial) for serious infections such as those caused by *A. pleuropneumoniae* (Prescott, 2006). This could be interesting to investigate, but it should be remembered that the treatment strategies employed in paper II and paper III corresponded to the recommendations of the manufacturer (FASS vet., 2010).

Acquired resistance should always be considered in cases of treatment failure and resistance of *A. pleuropneumoniae* to penicillin has been reported for a number of years from different countries across the world (Hendriksen *et al.*, 2008; Matter *et al.*, 2007; Gutierrez-Martin *et al.*, 2006). However, all 147 samples of *A. pleuropneumoniae* submitted to SVA since 2005 have had MIC values $\leq$ 0.5 mg/L and there are no signs of an acquired resistance to penicillin (SVARM, 2010). The strain used for the inoculation in the experimental trial had an MIC value of 0.5 mg/L (VetMIC, SVA) why this strain should be regarded as sensitive to penicillin.

Bearing the above in mind, it should be remembered that penicillin has been used with good results for treating acute actinobacillosis in commercial pig herds[2], and penicillin has often been considered to be the first choice antimicrobial substance in antibiotic policy documents. Possibly, the reported efficacy of penicillin in commercial pig herds could be due to pigs being exposed to lower infectious doses compared to the inoculation dose used in the experimental trial (Paper II and III). Another explanation could be that unifactorial infections are rare in practice, as was indicated by the serological responses to *P. multocida* (Paper I). Swedish isolates of *P. multocida* are generally sensitive to penicillin, which could contribute to the treatment efficacy reported for penicillin in treating commercial pigs for respiratory disease symptoms.

Still, the results indicate that it could be questioned if penicillin is the drug of choice when combating acute *A. pleuropneumoniae* infections in pig herds. Instead tetracycline appears to be an interesting alternative, due to good

[2] Magnus Paulsson, personal communication

clinical efficacy, simultaneously allowing a protective immune response to develop (Papers II and III). The latter is of great interest in practice, as pigs are likely to be re-exposed to the microbe.

Enrofloxacin had a superior efficacy (Papers II and III), but as pigs did not mount a protective immunity when treated at an early phase of the infection, the drug ought to be saved for treating severely affected animals. It could be argued that pigs in commercial herds will probably be treated at a later phase of the infection than in the trial presented. The results of the tetracycline treated pigs (Papers II and III) indicate that pigs could then develop a protective immunity, even when treated with enrofloxacin. This is an interesting thought, and ought to be investigated further. Still, enrofloxacin should be used judiciously in line with prudent use of antimicrobials. Not the least since enrofloxacin, due to its superior efficacy ought to be the drug of choice in eradication attempts, and when performing other strategic measures with the aim of reducing the pathogen load of *A. pleuropneumoniae* to a minimum in populations known to have developed a protective immunity to the microbe.

The first study and the experimental trial both indicated that pigs that had developed an immune response to *A. pleuropneumoniae* also developed partial or total immunity towards reinfections with *A. pleuropneumoniae* (Papers I, II and III). This was also in accordance with a number of other studies performed over the years which have demonstrated that pigs which were exposed to a natural infection were immune to subsequent reinfections (Krejci *et al.*, 2005; Nechvatalova *et al.*, 2005; Cruijsen *et al.*, 1995a; Cruijsen *et al.*, 1995b; Cruijsen *et al.*, 1995c; Loftager *et al.*, 1993; Bosse *et al.*, 1992). Therefore, vaccinations have been considered a tempting way to prevent actinobacillosis, but so far have different vaccines and vaccination strategies not been very successful (Jirawattanapong *et al.*, 2008; Ramjeet *et al.*, 2008; Haesebrouck *et al.*, 2004; Beskow *et al.*, 1993; Beskow *et al.*, 1989).

The general aim with vaccinations is to establish a level of immunity so that pigs can withstand an infection without becoming diseased. When pigs from different sources are allocated to a fattening unit, transmission of *A. pleuropneumoniae* from subclinical carriers is likely to occur (Velthuis *et al.*, 2002; Nielsen *et al.*, 2000; Savoye *et al.*, 2000; Wallgren *et al.*, 1993; Artursson *et al.*, 1989). This also applies to pigs from a single source if they are allocated to facilities housing several different age groups as transmission

of *A. pleuropneumoniae* may occur between units even though each age group is reared on an all-in all-out basis (Nielsen *et al.*, 2000). Therefore vaccinations against *A. pleuropneumoniae* were investigated in a fattening herd affected first by subclinical infections with *A. pleuropneumoniae* reflected in the slaughter registrations and then later by acute outbreaks of actinobacillosis. Prior to the initiation of the vaccinations, it was concluded that the pigs were seronegative to *A. pleuropneumoniae* on arrival to the herd at an age of approximately nine to 12 weeks. Therefore, pigs were not considered to have an active infection on arrival. However, as stress may cause a subclinical infection to be activated (Roth & Thacker, 2006), there may have been an increase in transmission after arrival to the fattening herd.

When pigs were vaccinated twice, on arrival and with a booster dose given 28 days later, the vaccine failed to induce a protective immunity to *A. pleuropneumoniae* (Paper IV). Therefore, a three-dose vaccination protocol was commenced (on arrival and 28 and 56 days later). Also this vaccination scheme failed to protect pigs from developing clinical actinobacillosis. The purpose of the three-dose protocol was to prolong the period of protection induced by the vaccine as the protection is claimed to be of short duration (FASS vet., 2010).

Another potential cause for the failure of the vaccine to induce a protective immunity in pigs receiving the first vaccination on arrival at the fattening herd may be that the pigs were vaccinated too late when they had already become infected. However, this was not believed to be the case, because pigs were generally seronegative on arrival and it has been demonstrated that a protective immunity can develop in pigs in spite of the presence of both passively derived and actively formed antibodies (Krejci *et al.*, 2005). A three-dose vaccination protocol against actinobacillosis using the same vaccine as used in this vaccination trial also failed to prevent actinobacillosis although the first vaccination was performed at the age of six weeks according to the manufacturer's instruction (Jirawattanapong *et al.*, 2008). By vaccinating at the age of six weeks, piglets were allowed time to mount an immune response before they were likely to become infected. However, as piglets may become colonized already at 11 days of age, the vaccination time point may not be crucial for the development of a protective immunity (Vigre *et al.*, 2002).

It appears that the vaccine used failed to trigger the essential parts of the immune system in order to induce protective immunity. It appeared that the

Apx toxins did not induce protective immunity when applied together with an OMP intra muscularly and that the presence of antibodies against LPS is indicative of protection if these antibodies are not protective in themselves. In this context, it could be argued that the antigen of the ELISA employed contained no protein and therefore only measured antibodies to LPS and CPS. As the vaccine used was based on the OMP and three toxoids (ApxI, ApxII and ApxIII), the ELISA did not detect antibodies induced by the vaccine itself. However, as clinical problems induced by *A. pleuropneumoniae* were continuously recorded in the herd, the vaccine failed to induce sufficient protection against *A. pleuropneumoniae* infections. At the first acute outbreak of actinobacillosis, a subsequent increase in antibody titers to *A. pleuropneumoniae* was detected with the LPS-based ELISA. This indicates the presence of the microbe in the herd which was confirmed as *A. pleuropneumoniae* serotype 2 was isolated at necropsy.

The intra muscular application of the vaccine may not have induced local immunity which is considered likely to play an important role in the defence against *A. pleuropneumoniae* infections. Perhaps other routes for administration or other antigenic preparations could be more efficient. An intra dermal administration route which induces both mucosal and cell-mediated immune responses has shown promising results (Bernardy *et al.*, 2008). Also, the use of live attenuated vaccines which mimic the natural course of an infection with *A. pleuropneumoniae* has a higher potential of conferring protective immunity against acute actinobacillosis (Maas *et al.*, 2006).

Regardless, the pathogen load was reduced as registrations for pleuritis recorded at slaughter decreased from 25% to 5% over the two-year period when the study was performed although mortality rates and weight gains remained constant over time. It was striking that the incidence of pleuritis registered at slaughter decreased after terminating the vaccinations – both when pigs were vaccinated twice and when they were vaccinated three times. Thus, the vaccinations may have contributed to a decrease in the pathogen load of the herd, but the increased awareness among the staff for clinical signs of respiratory disease probably lead to an increased incidence of treatments that in turn certainly contributed significantly to the positive results.

Weight gain is generally considered to reflect the health status of pigs well (Wallgren *et al.*, 1993), and the influence of subclinical infections is immense

(Wallgren, 2000). However, it may be difficult to demonstrate the effect of subclinical infections on growth in commercial herds (Andreasen *et al.*, 2001; Regula *et al.*, 2000; Rohrbach *et al.*, 1993; Hunneman, 1986). In accordance with this, no differences in growth rate between unvaccinated or vaccinated groups were recorded in the vaccination trial (Paper IV).

Still, differences in DWG over time were observed in the herd considered to be endemically infected with *A. pleuropneumoniae* (Paper I). A reduction in weight gain was observed between the age of nine and 10 weeks which was followed by seroconversion at the age of 11 weeks. This reduction in weight gain most probably reflected the infection with *A. pleuropneumoniae*, but may also have been influenced by the "stress" imposed on the pigs as they were transferred to the weaning unit at this age. "Stress" is known to reduce growth rate (Rutherford *et al.*, 2006; Sutherland *et al.*, 2006; Hyun *et al.*, 1998), but as "stress" also impairs immune functions (Salak-Johnson & McGlone, 2007), infections are more likely to affect pigs under "stress". If pigs are subjected to "stress" at a time when passive immunity has subsided to low levels and they are not yet capable to mount a mature immune response to the particular pathogen, they would be even more prone to become infected and develop disease.

Interestingly, there were some unexpected observations regarding the growth rate of the offspring to sows with low or high levels of serum antibodies to *A. pleuropneumoniae* (Paper I). Apart from the expected growth retardation seen at the time of seroconversion, reductions in weight gain were also observed on another two occasions further on during the rearing period. The reduction observed between the age of 12 and 13 weeks was believed to be due to crowding, as DWG increased after the transfer to larger pens in the same unit and there was no concurrent increase in serum antibodies indicating a re-exposure to *A. pleuropneumoniae*. Space allowance has previously been shown to affect weight gain (Brumm, 2004; Wolter *et al.*, 2002). The increase in DWG observed after the transfer to the fattening unit despite the concurrent increase in serum antibodies to *A. pleuropneumoniae* further strengthens the assumption that crowding caused the reduced growth rates observed prior to the transfers. It also indicates that the pigs had developed a partial or complete resistance to the reinfection with *A. pleuropneumoniae* when they were transferred to the fattening unit with continuous production (Paper I). Also, the lymphocyte stimulations demonstrated that an exposure to *A. pleuropneumoniae* generated an

activation of the humoral and cellular immune systems which implies that pigs can develop resistance to reinfections (Paper III).

Although there was a difference in the serological responses to a natural infection with *A. pleuropneumoniae* in the offspring to sows with high or low levels of serum antibodies to *A. pleuropneumoniae*, this difference was not observed in the growth rate (Paper I). It has previously been demonstrated that pigs with a higher growth rate developed more severe clinical signs than pigs with moderate growth rate after a PRRS virus infection (Doeschl-Wilson *et al.*, 2009). In contrast, an increased growth rate has been reported among pigs consistently bred for high cellular and humoral immune responsiveness (Mallard *et al.*, 1998). A large variation in the immune responsiveness between litters has been reported (Edfors-Lilja *et al.*, 1994; Wallgren *et al.*, 1994), and perhaps, a difference in weight gain could have been recorded if the piglets had been selected based on the capacity of their own immune system instead of the level of serum antibodies to *A. pleuropneumoniae* of their dams.

On the other hand, acute infections with *A. pleuropneumoniae* definitely reduce growth (Wallgren *et al.*, 1999b; Wallgren *et al.*, 1999a) which was also confirmed in the experimental study (Papers II and III). Interestingly, there was also a reduction in DWG, even in the uninoculated pigs, following the first inoculation in the experimental trial (Paper II). This was believed to be caused by the frequent samplings (daily) performed during the first week after the inoculation as no clinical signs of respiratory disease were detected among the uninoculated pigs. As stated previously, "stress" has previously been shown to cause reductions in DWG (Hyun 1998, Rutherford 2006, Sutherland 2006).

Considering theses findings, it can be concluded that systemic antibodies appear to mirror the protection against infections with *A. pleuropneumoniae* well (Papers I, II and III). Further, the level of circulating antibodies to *A. pleuropneumoniae* in sows was concluded to be important for the level of protection transferred from sows to their offspring in colostrum (Paper I). Together, these findings suggest the possibility of increasing protection to *A. pleuropneumoniae* infections in both suckling piglets and growers. However, a similar protective immunity did not develop when fattening pigs were vaccinated against actinobacillosis using a commercially available vaccine (Paper IV). By studying the subsets of antibodies that are produced in response to infections with *A. pleuropneumoniae*, a more detailed picture of

the protective humoral immunity could have been obtained. Even more clues on how antibodies play a role in the protection against *A. pleuropneumoniae* infections could have been obtained if BALF had been analyzed.

Other components than antibodies are also likely to be important in acquiring a protective immunity to infections with *A. pleuropneumoniae*. The acute-phase protein responses and the leukocyte counts observed in pigs inoculated with *A. pleuropneumoniae* indicated that pigs had mounted a protective immunity when re-exposed to the same strain (Papers II and III). Also the lymphocyte stimulations indicated that the immune system had been activated by an exposure to *A. pleuropneumoniae* (Paper III). Interestingly, the stimulatory index of lymphocytes, as well as the pMBL response, was higher following the second exposure to *A. pleuropneumoniae*, regardless if pigs had been previously challenged and mounted a subsequent immune response to the previous inoculation (Paper III). This may indicate that the immune system had matured from the first to the second inoculation, *i.e.* between the age of 10 to 14 weeks. This would be in agreement with clinical observations in farrow-to-finish herds with high pathogen loads that have improved health status and performance by increasing the age for the relocation of the pigs to the fattening unit from 10-12 weeks of age to 14-16 weeks of age[3]. In agreement with this, an increased capacity of the immune system with age has been demonstrated in healthy piglets monitored from one to eight weeks of age (Grierson *et al.*, 2007). However, it should also be noted that no further improvement of the efficacy of the immune system was observed after ten weeks of age when pigs were followed until they were 15 weeks old (Juul-Madsen *et al.*, 2010).

Also other parts of the immune system, not included in these studies, may be equally important for the protection against *A. pleuropneumoniae* infections. Therefore further studies of the immune response to *A. pleuropneumoniae* infections are needed, not the least with the aim of developing vaccines which will mimic a natural infection with *A. pleuropneumoniae* well enough to induce partial or complete protectin against actinobacillosis.

[3] Wallgren, unpublished data

5 Conclusions

- Serum antibodies to polysaccharides of *A. pleuropneumoniae* appear to play an important role in the protection against *A. pleuropneumoniae* infections.
- There are indications that protection against infections with *A. pleuropneumoniae* may be enhanced in piglets by improving serum antibody levels of sows. This could possibly be achieved by vaccinations of sows, as levels of serum antibodies to *A. pleuropneumoniae* in sows are reflected in colostral levels. Sows with high levels of serum antibodies show high levels of colostral antibodies.
- Offspring to sows with high levels of antibodies to *A. pleuropneumoniae* had higher levels of serum antibodies that persisted for a longer period of time compared to piglets from sows with lower levels of antibodies. Thus, these piglets are more likely to be better protected against infections with *A. pleuropneumoniae*.
- Offspring to sows with high levels of antibodies to *A. pleuropneumoniae* produced higher concentrations of antibodies to *A. pleuropneumoniae* when they seroconverted. By vaccinating piglets, immunity on herd level could be enhanced with fewer piglets being susceptible to disease when they encounter the pathogen. This is likely to occur following the transfer to a fattening unit.
- When treating clinical cases of actinobacillosis, attention should be paid to the severity of disease symptoms, the number of affected animals and if pigs are likely to re-encounter infections with *A.* pleuropneumoniae when selecting the drug for treatment. Treatment with enrofloxacin may be required for pigs with severe clinical symptoms as they are otherwise likely to die. However, if pigs risk to be reinfected at a later point during the rearing period, tetracycline may be the drug of choice. Another advantage with tetracycline is that it can be used for group-medication should many pigs be affected. Penicillin treatments of infections with *A. pleuropneumoniae* are questionable using the recommended doses and dose intervals.
- Vaccinations against actinobacillosis may reduce the pathogen load over time as indicated by a reduced incidence of pleuritits recorded at slaughter in a specialized fattening herd. However, the vaccine commercially available today did not induce protective immunity in itself. Various preventive measures and medical treatments are therefore still of importance to prevent clinical disease and to reduce the incidence of pleuritis recorded at slaughter.

6 Implications for future research

- Identify key virulence factors for *Actinobacillus pleuropneumoniae* in order to be able to develop vaccines conferring protection against infections with *A. pleuropneumoniae*.

- Investigate how different routes of vaccine administration influence the development of systemic and local immunity so that vaccines inducing appropriate local and systemic protective immunity can be developed.

- Investigate the possibility of vaccinating sows in order to obtain a higher level of protection for the piglets under a longer period of time which may also reduce the number of subclinical carriers.

- Investigate in more detail immunological parameters in offspring to sows with high and low amounts of antibodies to *A. pleuropneumoniae*.

- Extend the studies on the long-term effects of treatment strategies by investigating if and how the time point for initiating enrofloxacin treatments of *A. pleuropneumoniae* infections will influence the development of disease and the immune response.

7 Populärvetenskaplig sammanfattning

7.1 Bakgrund

Actinobacillus pleuropneumoniae är en bakterie som kan ge upphov till allvarliga lunginfektioner hos grisar. Den allvarligaste formen, så kallad elakartad lungsjuka, förlöper ofta med dödlig utgång. Grisar som insjuknar i elakartad lungsjuka upphör att äta, får hög feber, hosta och andnöd men kan även dö utan föregående symtom. Vanligare är dock en kronisk form där sjukdomen inte ger några märkbara tecken på sjukdom utan ofta upptäcks som mer eller mindre utbredda brösthinneinflammationer i samband med besiktningen vid slakt.

Sedan 1986, då rutinmässig användning av så kallade tillväxtbefrämjande tillsatser av antibiotika i fodret till livsmedelsproducerande djur förbjöds, har aktiva insatser utvecklats för att minska sjukligheten vilket har bidragit till det gynnsamma hälsoläge som råder inom svensk grisproduktion. Bland annat infördes omgångsproduktion från födsel till slakt i större omfattning. Omgångsproduktion innebär att avkommorna till en grupp suggor som grisar in under cirka en veckas tid föds upp tillsammans utan att blandas med grisar av annan ålder. Denna åtgärd bidrog till att spridningen av luftvägsinfektioner minskade under 1990-talet.

Under senare år har dock trenden vänt och ett ökat antal registreringar av brösthinneinflammationer och fler akuta utbrott av elakartad lungsjuka har rapporterats. Bidragande orsaker till detta kan vara att besättningsstorleken ökat. Detta innebär att antalet mottagliga djur och antalet möjliga smittvägar ökar per besättning, liksom att konsekvenserna av ett sjukdomsutbrott blir större.

Därför har åtgärder vidtagits, såväl för att förhindra uppkomsten av problem som för att minska skadornas omfattning när infektionen har brutit ut. I ett akut skede används antibiotika för att behandla sjuka grisar. Individbehandlingar då grisarna sprutas med lämpligt antibiotikum används framför allt om enstaka grisar eller boxar har insjuknat. Har större delar av en omgång eller till och med en hel besättning drabbats medicineras grisarna vanligen i foder eller vatten.

All antibiotikaanvändning medför dock en risk för att resistens skall utvecklas. I Sverige förekommer idag ingen känd resistens mot de antibiotika som används för att behandla infektioner med *A. pleuropneumoniae*. Däremot har resistensutveckling blivit vanligare i andra länder.

Därför är det angeläget att förebygga uppkomsten av sjukdom. Förutom omgångsuppfödning har även ventilation, tvätt- och desinfektion mellan omgångar samt vaccination använts för att förebygga uppkomsten av sjukdom orsakad av *A. pleuropneumoniae*. Tyvärr har vaccination hittills inte visat sig vara särskilt effektivt för att minska uppkomsten av sjukdom. Däremot är grisar som har genomgått en infektion och överlevt skyddade mot återinfektioner. Därför var syftet med de undersökningar som utfördes inom ramen för detta avhandlingsarbete att öka kunskapen om hur immunförsvaret påverkas av infektionen och hur detta kan ha betydelse för grisens skydd mot nya infektioner.

7.2 Sammanfattning av studier och resultat

I den första studien som genomfördes i en konventionell, integrerad besättning undersöktes betydelsen av suggans immunitet för avkommornas skydd mot en infektion med *A. pleuropneumoniae* under uppväxten. Det visade sig att mängden antikroppar i blodet hos suggan även avspeglade sig i den mängd antikroppar som suggorna förde över till smågrisarna via råmjölken. Suggor som hade högre antikroppsnivåer i blodet hade även en större mängd antikroppar i råmjölken som dessutom avspeglade sig i den mängd antikroppar som kunde påvisas i smågrisarnas blod. Antikroppar kunde även påvisas under längre tid hos de smågrisar som hade fått en större mängd antikroppar via råmjölken. Detta tyder på att dessa smågrisar skulle kunna vara bättre skyddade mot en infektion med *A. pleuropneumoniae*. Dessa smågrisar bildade dessutom en större mängd antikroppar när de blev infekterade med *A. pleuropneumoniae* senare under uppfödningen.

Tidpunkten för infektionen återspeglade sig även i tillväxten som sjönk då. Däremot utvecklade inga av grisarna några allvarliga tecken på luftvägssjukdom vilket antyder att de utvecklade ett eget skydd mot infektionen.

I den andra studien undersöktes hur immunitetsutvecklingen påverkades av behandling med olika antibiotika efter en infektion med *A. pleuropneumoniae*. Grisarna infekterades återigen 28 dagar efter den första infektionen med samma stam av *A. pleuropneumoniae*. Denna gång utfördes inga behandlingar. Det visade sig att de grisar som behandlats med enrofloxacin tillfrisknade mycket snart efter behandling. De utvecklade ett akutfasproteinsvar men bildade inga antikroppar. När de senare utsattes för samma smitta på nytt utan samtidig behandling blev de lika sjuka som de grisar som infekterades utan att behandlas. De enrofloxacinbehandlade grisarna uppvisade återigen ett akutfasproteinsvar efter den andra infektionen. Detta svar var i paritet med det som sågs hos den grupp grisar som tidigare inte infekterats och som nu mötte infektionen för första gången. Djuren i båda dessa grupper bildade även antikroppar mot *A. pleuropneumoniae* denna gång.

De grisar som behandlades med tetracyklin svarade i princip lika bra kliniskt på behandling som de enrofloxacinbehandlade grisarna men till skillnad från dessa så påverkades de inte vid återinfektionen vilket även avspeglade sig i ett uteblivet akutfasproteinsvar efter återinfektionen. De penicillinbehandlade grisarna svarade däremot inte särskilt bra på behandling kliniskt sett utan blev lika sjuka som de grisar som infekterades utan att behandlas. Efter den första infektionen utvecklade dessa grisar såväl ett akutfasproteinsvar som antikroppar mot *A. pleuropneumoniae*. Därmed hade de utvecklat en skyddande immunitet vid tidpunkten för återinfektionen.

I den tredje studien undersöktes effekten av ett kommersiellt tillgängligt vaccin mot infektioner med *A. pleuropneumoniae* i en slaktsvinsbesättning som haft problem med anmärkningar för brösthinneinflammationer vid slakt och som också drabbats av ett akut utbrott av elakartad lungsjuka. Grisarna vaccinerades med ett kommersiellt tillgängligt vaccin vid ankomst till besättningen samt fyra veckor senare i ett första skede. Senare vaccinerades grisarna tre gånger med den första vaccinationen vid ankomst och de efterföljande två vaccinationerna med fyra veckors mellanrum. Tyvärr minskade inte förekomsten av elakartad lungsjuka hos de vaccinerade grisarna. Däremot sjönk antalet registrerade brösthinneinflammationer vid slakt markant med tiden. Studien visade att vaccinationerna inte skyddade

grisarna mot sjukdom men att smittrycket minskade över tid. Huruvida detta var en effekt av vaccinationerna kan inte säkert visas. Troligen kan en ökad uppmärksamhet bland personalen ha bidragit till att fler fall av akuta lunginflammationer upptäcktes med en ökad behandlingsfrekvens som följd. Sannolikt har den ökade frekvensen behandlingar bidragit till att minska smittrycket vilket visade sig i färre registrerade brösthinneinflammationer vid slakt.

Sammanfattningsvis kan sägas att förekomsten av antikroppar riktade mot *A. pleuropneumoniae* avspeglar immunitet mot sjukdom väl. Detta öppnar för möjligheten att öka skyddet hos både diande och växande grisar genom att höja nivåerna av cirkulerande antikroppar hos suggorna och därmed öka mängden antikroppar i råmjölken. Detta måste dock göras på ett kontrollerat sätt, exempelvis genom vaccination av suggor och förutsätter att vaccinationen leder till en antikroppsstegring och att dessa antikroppar blir skyddande. Framtagande av väl fungerande vacciner till växande grisar kan också utgöra ett alternativ för att kontrollera infektionen, dock måste vacciner som ger upphov till en skyddande immunitet utvecklas först.

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