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Actinobacillus pleuropneumoniae and the development of an ELISA for the detection of ApxIV antibody in swine oral fluids

Wendy Mecía González Guzmán
Iowa State University

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Actinobacillus pleuropneumoniae and the development
of an ELISA for the detection of ApxIV antibody in swine oral fluids

by

Wendy Mencía González Guzmán

A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

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Program of Study Committee:
Jeffrey J. Zimmerman, Major Professor
Timothy Frana
Ronald Griffith

Iowa State University

Ames, Iowa

2016

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DEDICATION

To my parents,
Bélgica and Juan Manuel

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ABSTRACT

Swine respiratory diseases are of major concern to pork producers because of economic consequences related to reduced productivity, increased mortality, and the higher costs associated with prevention, control, treatments, and diagnoses. *Actinobacillus pleuropneumoniae* (APP) is the causative agent of porcine pleuropneumonia, a respiratory disease found throughout the world. Pigs are the primary host of APP and infection can occur at any age. APP infections are often acute and rapidly fatal, but chronic and/or subclinical infections occur. In particular, the latter play a role in perpetuating endemic infections in populations.

A plethora of methods have been developed for the detection and/or diagnosis of APP, e.g., bacterial culture, antibody-based assays, and detection of nucleic acid targets using PCR. In particular, antibody detection provides an efficient, cost-effective approach for the surveillance of swine populations and several antibody assays have been developed, including ELISA assays based on polysaccharide antigen (capsular and LPS) and tests based on the detection of anti-toxin antibodies Apx I, ApxII, ApxIII, and ApxIV.

Research has shown that the use of oral fluid specimens in diagnosis provides several advantages compared serum, including easier sample collection and better herd-level sensitivity and specificity (Olsen et al., 2013). Within this context, the focus of this thesis was on the development of an antibody ELISA for the detection of *Actinobacillus pleuropneumoniae* (APP) ApxIV toxin antibodies in swine oral fluid specimens.

As described in Chapter 3, antibody responses specific for ApxIV in serum and oral fluid were compared in pigs inoculated with APP serovars 1, 5, 7, or 12 under experimental conditions using a commercial ApxIV antibody ELISA. The detection of antibodies in the oral fluid was achieved by adapting the serum ELISA protocol was adapted to the oral fluid matrix, as has been previously described (Kittawornrat et al., 2013). Serum samples were collected weekly and oral fluid samples were collected daily from individual pigs from day post inoculation (DPI) -14 through DPI 56. The LPS ELISA serum response showed that all

pigs exposed to serovars 1 and 7 were positive from DPI 14 through DPI 56, with some animals demonstrating a LPS-specific IgG response as early as 7 DPI. In contrast, the LPS antibody response was transient or absent in pigs inoculated with serovars 5 and 12, suggesting that inoculation of pigs did not result in infection. Both IgM and IgG ApxIV serum antibody was detected in animals infected with serovars 1 and 7. Likewise, oral fluid samples from these animals showed a significant ($p < 0.05$) ApxIV IgG response. Thus, this pilot experiment suggested that ELISAs based on the detection of ApxIV IgG antibody in oral fluid samples could be developed. Future work will be required to establish the ApxIV oral fluid ELISA cutoff and evaluate the application of the assay in the field.

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CHAPTER 1. THESIS ORGANIZATION

This thesis consists of 3 chapters. The Chapter 1 describes the organization of the thesis and Chapter 2 is a review entitled “An update on *Actinobacillus pleuropneumoniae*”. Chapter 3, “Detection of *Actinobacillus pleuropneumoniae* ApxIV toxin antibody in serum and oral fluid specimens from pigs inoculated under experimental conditions” was submitted for publication in the Journal of Veterinary Diagnostic Investigation. The tables and figures associated with each chapter follow the references. The final chapter contains the General Conclusions of the thesis.

CHAPTER 2. AN UPDATE ON *ACTINOBACILLUS PLEUROPNEUMONIAE*

Wendy Mencía González Guzmán, DVM

INTRODUCTION

The genus *Actinobacillus* is a small, gram-negative, pleomorphic, non-spore-forming, coccoid-to-rod-shaped group of bacteria in the *Pasteurellacea* family. The genus is facultatively anaerobic and has complex nutritional requirements (MacInnes and Bossé, 2004). The bacteria in genus *Actinobacillus* are always associated with mucous membranes and have a limited host range (MacInnes and Lally, 2006).

Actinobacillus pleuropneumoniae (APP) was first described as the cause of pneumonic lesions in pigs experimentally inoculated with classical swine fever virus and was classified at that time as a *Haemophilus*-like bacterium (Pattison et al., 1957). Later, an investigation of acute, fatal, respiratory disease in swine farms in Argentina led to a bacterium identified as *Haemophilus pleuropneumoniae* (Shope, 1964a). *H. pleuropneumoniae* was also shown to cause acute pneumonia when inoculated into pigs under experimental conditions (Shope et al., 1964b). Because of its growth requirements and hemolytic characteristics, APP was originally considered a member of the *Haemophilus* organisms of humans and thought to be synonymous with *H. parahaemolyticus* (Zinnemann, 1971). Killian (1976) separated this bacterium from the human *Haemophilus* spp. on the basis of biochemical characteristics, phenotypic differences, and its pathogenicity for swine. Later, Kilian et al (1978) proposed the name originally given by Shope (1964a) for the causal agent of porcine pleuropneumonia. The organism was finally placed in the genus *Actinobacillus* based on DNA homology studies (Pohl et al., 1983).

Two APP biotypes have been identified on the basis of the nicotinamide adenine dinucleotide (NAD or V-factor) requirement for growth: biotype I (NAD-dependent) and biotype II (NAD-independent) (Pohl et al., 1983). Based on the composition of the capsular polysaccharide (CPS), the two biotypes have been divided into 15 serovars. Biotype I includes 13 serotypes (1-12, 15) and biotype II contains 2 serotypes (13-14) (Blackall et al.,

2002; Perry et al., 1990). Serotypes 1 and 5 are further differentiated into 1a and 1b, and 5a and 5b, based on minor differences in the polysaccharide structures (Jolie et al., 1994; Nielsen, 1985). Recently, a 16th serotype was proposed following the recovery of an APP isolate that did not fit the current classification criteria from swine with pleuropneumonia (Sarkoezi et al., 2015).

Closely related bacterial species also are found in swine and must be differentiated from APP. *Actinobacillus suis*, first described in 1962, is an early colonizer of the upper respiratory tract of swine (van Dorssen and Jaartsveld, 1962; MacInnes and Lally, 2006). *A. suis* is usually associated with septicemia and sudden death in young piglets, but may also be found in weaned pigs (Gottschalk, 2012). It shares characteristics with APP, including iron binding proteins (TbpA and TbpB), urease positivity, and the production of RTX toxins (ApxI and ApxII) (MacInnes and Bossé, 2004).

More recently, Gottschalk et al (2003) reported the description of two APP-like isolates (9953L55 and 0347) that were antigenically and biochemically similar to APP serovars 1 and 9, respectively, but did not produce clinical disease or lesions after experimental inoculation of pigs and were genetically distinct from APP. On that basis, a new *Actinobacillus* species was proposed (*Actinobacillus porciconsillarum*). *A. porciconsillarum* has since been identified in Spain (Martinez and Maldonado, 2006), Thailand (Tonpitak et al., 2007), and Japan (Ohba et al., 2007).

SUSCEPTIBLE SPECIES

Pigs are the primary host of APP and infection can occur at any age (Gottschalk, 2012). APP is an obligate parasite and is typically isolated from lungs, nasal secretions, and tonsils of infected pigs (Auger et al., 2009; Chiers et al., 1999; Dom et al., 1994; Gottschalk, 2012). However, there are a few reports describing the recovery of APP from other sites, e.g., secretions from pigs with otitis media (Duff et al., 1996) and from the joints of pigs with osteomyelitis (Jensen et al., 1999).

APP infections are not limited to swine in commercial production settings. Thus, Štukelj et al (2014) reported 28.3% of 184 samples collected from wild boar (*Sus scrofa*) in Slovenia were seropositive to APP. Likewise, Marinou et al (2015) reported that 90.5% of the samples collected from wild boar in Greece were seropositive for APP. In the United States, Baroch et al (2015) found that 69.7% of the feral swine sampled were serologically positive for APP. Cumulatively, these reports suggest that APP infections are common in feral swine. For that reason, feral swine should be considered a reservoir of APP and a potential source of infection for domestic swine.

Although swine are the natural host, APP has occasionally been recovered from cattle, deer, and sheep (see reviews by Desrosiers et al., 1998 and Dubreuil et al., 2000). Hervás et al (1996) reported that APP was associated with pneumonic lesions in lambs in Spain. Pérez et al (2014) reported the detection of APP by PCR in layer hens with signs of infectious coryza (*Avibacterium paragallinarum*). These reports are of interest, but they are not of sufficient weight to support the concept that non-porcine species are significant in the epidemiology or ecology of APP.

VIRULENCE FACTORS AND PATHOGENESIS

As general rule, bacteria cause disease by two mechanisms: tissue invasion and toxin production (Post, 2012). The virulence of *A. pleuropneumoniae* is multifactorial and there are several products (secreted factors) as well as bacterial structures involved in its pathogenesis. The role of APP virulence factors in these mechanisms has been reviewed in detail elsewhere (Bossé et al., 2002; Chiers et al., 2010). A summary of the major contributors to APP pathogenesis is presented below.

Surface polysaccharides

The capsular polysaccharide (CP) is a major structural cell component and the basis for serovar specificity (Inzana, 1991; Perry et al., 1990). The CP is considered an important virulence factor in APP infections and is involved in protecting the bacteria from antibody and preventing phagocytosis (Inzana et al., 1988; Rioux et al., 2000; Ward et al., 1994). That

is, greater production of CP by an APP strain is correlated with more extensive pulmonary lesions and greater resistance to killing (Bandara et al., 2003; Ward and Inzana, 1997). Interestingly, Van Overbeke et al (2002) reported that APP growth conditions altered the production of CP, which in turn affected the expression of surface antigens involved in the adhesion.

Lipopolysaccharide (LPS) is a complex biomolecule divided into three regions: lipid A, anchored in the outer membrane; the core oligosaccharide, and the O-antigen, a polysaccharide chain composed of repeated units (Wang and Quinn, 2010). Three types of APP LPS structures have been described: smooth (serovars 2, 4, and 7), rough (serovars 3 and 6), and semi-rough (serovars 1 and 5). This results in differences in the gross appearance of colonies (Byrd and Kadis, 1989). Although the composition of LPS differs among APP serovars, antibody cross-reactions occur among serovars 1, 9, and 11; 3, 6, and 8; and 4 and 7 because of similarities in the O-antigen (Perry et al., 1990).

LPS has been shown to play a role in the adherence of APP to the epithelial cells of the respiratory tract (Bélanger et al., 1990; Paradis et al., 1999) and contributes to APP-associated respiratory disease by enhancing the effect of Apx toxins on phagocytes (Ramjeet et al., 2005). Bélanger et al (1990) reported that adhesion of APP to tracheal epithelium was higher in isolates with a smooth versus semi-rough LPS. Other studies demonstrated that the LPS outer core affected APP adhesion and virulence (Ramjeet et al., 2005; 2008a). Specifically, the absence of terminals for galactose (Gal I) and D, D Heptose (Hep IV) in the outer core resulted in the loss of the ability to adhere to respiratory tract cells and greater susceptibility to antimicrobial peptides (Ramjeet et al., 2005).

The formation of biofilms has been demonstrated in APP isolates and reportedly has relevance to colonization and pathogenesis (Kaplan and Mulks, 2005). Recent work demonstrated that the lack of O-antigen reduced the ability of APP to form biofilms (Hathroubi et al., 2015). More specifically, the deletion of genes involved in biofilm formation (*arcA*, *luxS*, *hns*) resulted in the attenuation of virulence (Buettner et al., 2008; Dalai et al., 2009; Li et al., 2008). As discussed by Chiers et al (2010), biofilms increase

APP resistance to antimicrobials agents and to the host's immune response by interfering with macrophage phagocytic activity and inhibiting antibody access to the cell surface.

Exotoxins

Four APP exotoxins (Apx I, II, III, and IV) have been described (Frey, 1995; Schaller et al., 1999). These RTX (**R**epeats in the structural **T**o**X**in) pore-forming toxins have cytotoxic or cytolytic effects on epithelial cells, endothelial cells, red blood cells, neutrophils, and macrophages (Frey, 1995, 2011; Schaller et al., 2000). Not all serovars produce all toxins (see Table 2); with the exception of ApxIV, which is produced universally. ApxIV is generally thought to be produced only during infection (Deslandes et al., 2010; Schaller et al., 1999), but a study analyzing a DIVA subunit vaccine reported the expression of the ApxIVA gene under *in vitro* conditions (Buettner et al., 2011). Although their exact contribution to the pathogenesis of APP is not clear, virulence is related to Apx production (Beck et al., 1994; Frey, 1995). In general, Apx exotoxins are thought to be involved in the evasion of host defense mechanisms and in the induction of lung lesions (Dom et al., 1994; Frey, 2011; Haesebrouck et al., 1997; Kamp et al., 1997).

Transferrin binding proteins

If unable to acquire sufficient iron, APP will be unable to grow and will be eliminated by host defense mechanisms or succumb to nutrient deficiency (Ratledge and Dover, 2000). The concentration of iron in the extracellular environment of the host is not adequate, but APP has developed compensatory mechanisms for iron acquisition (Ratledge and Dover, 2000). Lipid A of the LPS has been associated with the nutrient acquisition, specifically with iron uptake (Bossé et al., 2002; Chiers et al., 2010). In addition, APP expresses two transferrin-binding proteins (TbpA and TbpB) that enable it to use porcine transferrin as source of iron (Baltes et al., 2002). Several studies have reported that these transferrin binding proteins play a role in APP virulence (Baltes et al., 2002; Bossé et al., 2002; Chiers et al., 2010; Haesebrouck et al., 1997).

Other factors

As reviewed elsewhere, (Bossé et al., 2002; Chiers et al., 2010; Haesebrouck et al., 1997; Inzana, 1991), a number of outer membrane proteins are thought to contribute to virulence. Some have been shown to play a role in the adhesion of APP to the respiratory tract (Baltes and Gerlach, 2004; Mullen et al., 2008) and others to the acquisition of the nutrients necessary for proliferation (Deneer and Potter, 1989; Lone et al., 2009). Additionally, type IV fimbriae have been observed in APP isolates (Dom et al., 1994; Zhang et al., 2000). This type of fimbriae has been associated with adhesion, colonization and survival of APP in the porcine respiratory tract (Boekema et al., 2004; Deslandes et al., 2010; Negrete-Abascal et al., 2003).

Differences in virulence among and within serovars

All APP serovars are considered pathogenic, i.e., capable of causing disease and mortality, but differences in virulence are observed among and within serovars (Frey, 1995). In general, serovars 1, 5, 9, and 11 are considered the most virulent; serovars 2-4, 6-8, 12, and 15 are considered moderately virulent; and serovars 10, 13, and 14 the least virulent (Frey, 2003; Komal and Mittal, 1990). Differences in virulence are primarily associated with the specific bacterial structures discussed above and RTX exotoxin production patterns (Beck et al., 1994; Frey, 2003; Frey and Nicolet, 1990). Production of ApxI and ApxII are considered to be primarily responsible for full APP virulence (Reimer et al., 1995), but Kamp et al (1997) demonstrated that ApxI and ApxIII were involved in the production of clinical signs and lung lesions for pleuropneumonia. ApxIV, which is produced by all APP serovars, is considered necessary for the full expression of virulence, through mechanism(s) that have not yet been described (Liu et al., 2009). Thus, the expression of more than one Apx, in addition to ApxIV, is correlated with the increased virulence (Beck et al., 1994; Frey, 1995, 2003).

GEOGRAPHIC DISTRIBUTION

The geographic distribution of APP serovars is summarized in Table 1. As reviewed by Gottschalk (2012), APP infections in pigs have been reported in Europe, Asia, North America, Latin America, and Australia. Although poorly documented, APP infections are

reported to occur in Africa, as well (Veary, 1989). Thus, APP infections occur wherever swine are found.

As discussed by Gottschalk (2015a), information concerning the country-specific distribution of APP serovars is lacking or out of date, despite the fact that knowing which serovars are present in a region is essential information for the diagnosis and control of the disease. In general, APP biotype II is associated with the majority of outbreaks in Europe, while APP serovars belonging to biotype I are more common in America and Asia (Gottschalk, 2012).

Within any region, the predominant APP serovars may change over time. For example, serovars 2 and 5 were the most common in Korea between 1995 and 2010 (Yoo et al., 2014), but Lee et al (2015) recently reported that serovar 1 and 5 were dominant in Korea, i.e., serovar 1 had replaced serovar 2. Lee et al (2015) hypothesized that this change could be due to intensive serovar-specific vaccination. That is, vaccination may decrease APP-related morbidity and mortality, but does not provide cross-protection to multiples serovars. In a similar vein, MacInnes et al (2008) and Gottschalk and Lacouture (2015b) reported a reduction in infections with APP serovars 1 and 5 over time and an increase in infections with serovars 7 and 12 in Ontario (Canada) herds. The authors suggested that this change resulted from improved farm management, e.g., more testing and monitoring, better biosecurity, more all-in/all-out management, and less commingling of animals from multiple sources. In addition, this change may reflect the impact of a program for the control of APP serovars 1 and 5 in breeding stock implemented by the provincial government of Ontario.

IMPACT ON SWINE HEALTH

The clinical expression of APP ranges from peracute death to chronic subclinical infection depending on the serovar, the immune status of the host, and exposure dose (Bossé, 2002; Deslandes, 2010; Gottschalk, 2012; MacInnes and Bossé, 2004; Marsteller and Fenwick, 1999). APP infections can also affect pig health through interactions with other factors (Bochev, 2007). For example, subclinical APP can disrupt normal host metabolic processes and optimal nutrient utilization, thereby affecting pig performance parameters, e.g., weight

gain, pregnancy, lactation, and others (Dial, 2002). Thus, the economic impact of APP in commercial swine production may be the result of acute mortality (often in late finishing), a reduction in productivity parameters, and costs associated with diagnostics and the implementation of control measures (Gottschalk, 2012; Losinger et al., 1998). Losinger (2005) estimated the cost of measures for the control of APP infections in the U.S at \$32 million dollars in 1995 (more recent estimates are not available).

In reality, estimates of the economic impact of APP on swine production are confounded by the fact that APP commonly occurs concurrently with other respiratory infections in the “porcine respiratory disease complex” (PRDC) (Thacker and Thanawongnuwech, 2008). PRDC is defined as a multifactorial respiratory disease involving both bacterial and viral agents in grower-finisher pigs and characterized by decreased growth rates, reduced feed-conversion, anorexia, fever, cough, and dyspnea (Brockmeier et al., 2002; Hansen et al., 2010; Opriessnig et al., 2011). The distribution and severity of the disease is thought to involve several predisposing determinants including interactions among infectious agents, environmental factors, production and management factors, and host determinants, e.g., immune status and age (Bochev, 2007; Opriessnig et al., 2011). Infectious agents involved in PRDC can include primary pathogens, i.e., those agents capable of causing disease as single infections, and opportunistic pathogens that interact synergistically with primary pathogens (Brockmeier et al., 2002). Depending on the region, primary viral agents may include porcine reproductive and respiratory syndrome virus (PRRSV), porcine circovirus type 2 (PCV2), swine influenza virus (SIV), classical swine fever (CSF), pseudorabies virus (PRV), and paramyxoviruses (PMV) (Thacker, 2008; Bochev, 2007; Opriessnig et al., 2011). Primary bacterial pathogens can include *Mycoplasma hyopneumoniae*, *Bordetella bronchiseptica*, and *Actinobacillus pleuropneumoniae* (Thacker, 2008; Brockmeier et al., 2002).

TRANSMISSION

Transmission from the pathogen’s perspective is the successful completion of a series of challenges that begin with the exit of an infectious agent from one host and end with its

replication in a new, susceptible host (Zimmerman, 2003). In the case of APP, transmission is considered to occur pig-to-pig, by infectious airborne droplets, or via fomites.

Transmission is not synonymous with disease. Thus, Hensel et al (1995) reported that experimental aerosol exposure to low concentrations of APP led to infection without clinical signs. Particularly for APP, respiratory disease is the result of interactions among a number of factors, not just with the presence of the agent (Stäk, 2000). This comment is germane because interpretation of the results of experiments attempting to understand the mechanisms of APP transmission can be affected by the serovar(s) included in the experiment, the immune status of the host, and the exposure dose (Bossé, 2002; Deslandes, 2010; Gottschalk, 2012; MacInnes and Bossé, 2004; Marsteller and Fenwick, 1999). APP's dose dependency is particularly challenging and transmission by experimental inoculation is a balance between achieving an infectious dose while avoiding excessively severe clinical signs and/or mortality (Sebunya et al., 1983).

Shedding of APP

APP colonizes the lower respiratory tract, but has also been shown to persist in the upper respiratory tract (Bossé, 2002; Chiers et al., 2010; Dom et al., 1994). There are no reports in the literature describing the detection of APP in semen, milk, feces, or urine.

Several studies suggested that tonsil colonization may play an important role in the transmission of the disease. Chiers et al (1999) found APP associated with the crypt wall epithelium of the pharyngeal tonsils and APP has been found in the tonsils of subclinically-infected pigs (Gottschalk, 2012). Velthuis et al (2002) showed that pigs carrying APP in tonsils were more likely to be infectious.

Shedding of APP through nasal secretions is considered a primary exit route for the bacterium (Gottschalk, 2012). In a transmission trial, Velthuis (2003) reported that the infectivity of APP was 6.13 times higher when more than 10 APP colonies were isolated from nasal swabs. In the field, Willson (1987) reported that the maximum isolation of APP from nasal secretions was in animals of 12 week of age; by the 15 weeks of age the bacteria could not be isolated. In a vaccination trial, Wongnarkpet et al (1999) reported peak

detection at 11 weeks of age and concluded that APP was more likely to be isolated from pigs at 4-11 week of age.

Exposure route and dose

Transmission of APP is believed to commonly occur via infectious airborne droplets over short distances between infected and susceptible pigs (Gottschalk, 2012; Sebunya and Saunders, 1983). Aerosol transmission of a disease can occur if two requirements are met: 1) an infectious aerosol is created and 2) susceptible animals inhale particles or droplet nuclei containing sufficient numbers of viable pathogen so as to achieve an infectious dose (Stärk, 1999). Airborne transmission of APP has been reported to occur over short distances on farms. Torremorell et al (1997) found evidence that aerosol-exposed animals were seropositive to APP serovars 1 and 5 and concluded that APP was transmitted by air over a distance of 1 meter. Similarly, Jobert (2000) reported the airborne transmission of APP serovar 9 over a distance of 2.5 meters. The question of airborne transmission of APP over longer distances has also been addressed. Kristensen et al (2004) found that APP airborne transmission between pig units was possible, but the probability of transmission was < 2% under field-like condition. Tobias et al (2014) quantified APP aerosol transmission in weaned pigs on a farm endemically infected with serovar 2 and found the transmission rate within pens was ten times higher than between pens.

In the field, APP transmission from infected sow to offspring occurs via close contact. Maternal immunity persists in piglets from 2 to 8 weeks (Crujisen et al., 1995; Gottschalk, 2012). For this reason, the appearance of clinical signs is unlikely at this age. Presumably, piglets that become infected from their dams become carriers and spread the infection post-weaning (Chiers et al., 2002; Gottschalk, 2012; Vigre et al., 2002).

Under experimental conditions, intranasal (IN) inoculation is a common route of exposure, but outcomes are variable, ranging from not achieving infection (Baarsch et al., 2000; Costa et al., 2011; Dom et al., 1994; Velthuis et al., 2002) to severe clinical signs, typical lesions of pleuropneumonia, and high mortality rates (Gómez-Laguna et al., 2014; Jobert, 2000; Marois et al., 2009; Tobias et al., 2014). The inconsistency observed with IN inoculation may be because some of the inoculum enters the esophagus, rather than the respiratory tract and/or

because the inoculum is given once, rather than the multiple exposures that occur in the field (Baarsch et al., 2000; Velthuis et al., 2002). Endobronchial inoculation has also been used under experimental conditions (Baarsch et al., 2000; Kamp et al., 1997).

Environmental stability

An experiment by del Río et al (2003) demonstrated the viability of APP in different transport systems stored at 4°C. In liquid media, APP was viable for 13 days in brain heart infusion and 23 days in horse serum with 0.01% NAD. In commercial transport systems designed for aerobic organisms, 54.2% of APP was viable after two days.

Loera-Muro et al (2013) reported the recovery of viable APP for at least 3 weeks at 20°C. Assavacheep and Rycroft (2013) reported the recovery of APP held at -20°C and -70°C for more than 17 weeks and concluded that viable APP could persist in frozen carcasses. They also demonstrated that the environmental stability of APP was improved in aqueous suspensions in the presence of NaCl and mucin, i.e., a model for nasal secretions. Under dry conditions, APP remained viable for more than 3 days in non-absorbent materials, suggesting a possible indirect transmission through fomites.

There are relatively few studies on viable APP in the farm environment. Recently, Loera-Muro et al (2013) reported the detection of APP in drinking water on swine farms by indirect immunofluorescence. The viability of the bacteria was confirmed by the Live/Dead® *BacLight* stain™ (Loera-Muro et al., 2013). Further, biofilm-like structures were found in the water using scanning electron microscopy. As reviewed previously, APP uses the biofilm structure to attach to surfaces and as strategy for survival in the environment (Jacques et al., 2010; Labrie et al., 2010). In a subsequent study, Loera-Muro et al (2014) found viable APP using a polymerase chain reaction test (PCR) in feed, air, and soil samples. Based on these findings it may be concluded that the environment can serve as reservoir for APP. In endemically-infected herds, the pattern of APP-associated clinical signs and mortality could be explained by increasing levels of environmental contamination over time and exposure of animals to successively higher doses.

Transmission within and between herds

The transmission of APP between herds is generally attributed to the introduction of carrier animals. However, research on the environmental stability of APP (reviewed above) also suggests that fomites (people, equipment, vehicles) could play an important role in the indirect transmission of APP between and within farms (Desrosiers and Moore, 1998). This is an area that merits more research.

Once APP enters a population, it circulates among animals by direct (nose-to-nose) and indirect (aerosols and fomites) transmission. Colonization of tonsils and excretion of APP from the upper respiratory tract is considered important for transmission (Chiers et al., 1999; Velthuis et al., 2002). Sows can infect their offspring during lactation, but infected pigs do not typically develop clinical disease until maternal antibodies wane at 6 to 12 weeks of age (Vigre et al., 2002). In the presence of maternal antibody, infected pigs replicate APP in nasal cavities and tonsils and nasal cavities become a source of infection (Chiers et al., 2001). These pigs do not always mount an immunological response against APP, which makes their detection more difficult (Chiers et al., 2010). Thus, animals that have survived the infection, animals that have been infected by exposure to low doses of the bacterium, or animals with maternal antibody can become subclinical carriers and silently spread the infection among the population (Velthuis et al., 2002).

Wildlife often plays a role in the diseases of domestic animals and this is probably true for APP, as well (Gortázar et al., 2007). In the U.S., Baroch et al (2015) reported the detection of serum antibodies against porcine reproductive syndrome virus (2.5%), porcine circovirus type 2 (25.3%), *Mycoplasma hyopneumoniae* (19.7%), and APP (69.7%) in samples from feral swine. Touloudi et al (2015) reported antibodies against porcine reproductive syndrome virus (12.8%), porcine circovirus type 2 (19.1%), and APP (57.4%) in wild boars in Greece. Similarly, Štukelj et al (2014) reported the detection of antibodies against Aujeszky's disease virus (45.1%), porcine circovirus type 2 (89.7%), *Mycoplasma hyopneumoniae* (15.8%), and APP (28.3%) in serum from wild boars in Slovenia. These studies support the concept that feral swine populations present an on-going risk for the introduction of APP and other infectious diseases into commercial swine populations (Corn et al., 2005, 2009).

PREVENTION AND CONTROL

Vaccines

A number of vaccines have been developed and used, but the prevention and control of APP through vaccination has been a challenge (Gottschalk, 2012; Haesebrouck et al., 2004; Ramjeet et al., 2008b). Several antigens have been evaluated as vaccine candidates, including Apx toxins (Van Overbeke et al., 2001), LPS and capsular polysaccharide (Byrd and Kadis, 1992), outer membrane proteins (Oldfield et al., 2008), autotransporter proteins (Oldfield et al., 2009), DNA vaccines (Chiang et al., 2009; Lu et al., 2011), subunit vaccines (Buettner et al., 2011), and mutant vaccines (Inzana et al., 1993; Liu et al., 2007). Despite the many vaccines and vaccination protocols that have developed, there is not one vaccine that is efficacious against all serovars. Therefore it is recommended that the serovar present in the population be taken into account, if vaccination is used as a control measure (Vanni et al., 2012).

Antibiotics

If used sufficiently early in the course of the infection, antimicrobial agents have been used to control APP in pig production with relatively good success. However, even if treated, chronically-infected animals may not recover and will remain as carriers (Gottschalk, 2012; Willson and Osborne, 1985). A point of concern is that fact that increasing resistance of APP to antimicrobials has been reported. For example, Matter et al (2007) reported that APP isolates from slaughtered pigs were resistant to sulfamethoxazole, a sulfamethoxazole-trimethoprim combination, tiamulin, tilmicosin, tetracycline, penicillin, and ampicillin. Likewise, Vanni et al (2012) reported that APP isolates from farms in Italy were resistant to amoxicillin, amoxicillin/clavulanic acid, cefquinome, cotrimoxazole, penicillin G, ampicillin, and tilmicosin, and were decreasingly susceptible to gentamycin and marbofloxacin. These reports, particularly in the context of increasing restrictions on veterinary access to antimicrobial agents, are of serious concern.

Diagnosis and surveillance

The application of laboratory methods to the diagnosis and/or surveillance of APP have been extensively reviewed in several recent publications (Broes et al., 2007; Gottschalk, 2012, 2015a; Żmudzki et al., 2015). Herein we offer a brief summary of the main approaches used to identify APP infections.

Traditionally, the diagnosis of APP on farms has focused on the observation of clinical signs and the isolation of APP from tissues post mortem (Gottschalk, 2012). Detection may be adversely affected by sample transportation issues or with the technique used to grow the bacterium (Broes et al., 2007; Willson et al., 1987). However, this classic approach is useful when clinical signs are present and is likely to be successful when the bacterium is present in sufficient numbers in clinical specimens. This approach is not practical for identifying subclinical carriers and is not useful in monitoring progress in the clean-up of endemically-infected herds.

The use of antibody-based assays for detecting APP infections has become possible with the development of a variety of tests in recent years. These assays detect antibody against either polysaccharide antigens, capsular polysaccharide, lipopolysaccharide, or Apx toxins (Inzana and Fenwick, 2001; Gottschalk et al., 1994; Nielsen, 2000). In general, these techniques can be divided into those that are serovar or serogroup specific and those that are species-specific, i.e., capable of detecting antibodies against all serovars of APP (Bossé et al., 1990; Dreyfus et al., 2004; Eamens et al., 2012; Gottschalk, 1994; Grondahl et al., 2003).

Molecular techniques have been developed for the direct detection of APP (Cho and Chae, 2001; Fittipaldi et al., 2003), but limitations have been reported with these techniques due to challenges in analytical sensitivity and specificity. At this time, molecular techniques have primarily been used in research and not in routine diagnostics. Presumably, molecular diagnostics will become more common as the technology develops.

APP surveillance programs are the cornerstone for the control and/or eradication of the disease, but require the collection of samples from live pigs. Current diagnostic methods for

antibody detection or culture require the collection of samples from individual pigs (serum, nasal, tonsil, or endotracheal swabs). Alternatively, oral fluid specimens have proven to be useful for the detection of specific antibody and, therefore, highly suitable for the implementation of swine disease surveillance (Kittawornrat, et al., 2010; Ramirez et al., 2012; Prickett et al., 2008). In this thesis we explore the use of an ApxIV antibody ELISA for the detection of ApxIV antibody in oral fluids.

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Table 1. Geographic distribution of *Actinobacillus pleuropneumoniae* serovars by region

Region	Serovar	Reference
Eastern Europe	1-13	Kucerova et al., 2005; Shabunin et al., 2013; Štukelj et al., 2014; Žutić et al., 2014.
Western Europe	1-12	Chiers et al., 2002; Gutiérrez-Martín et al., 2006; Kamp et al., 1997; Nielsen, 1988; O'Neill et al., 2015; Sjölund, 2010.
Asia	1-3, 5, 7, 8, 12	Fukuyasu et al., 1996; Lee et al., 2015; Torres et al., 2008; Yang et al., 2011.
North America	1-3, 5-8, 10-13, 15	Fales et al., 1989; Gottschalk and Lacouture, 2015b; Gottschalk et al., 1994; MacInnes et al., 2008; Mittal et al., 1992; Ontiveros et al., 1995; Perry et al., 2012; Williams et al., 2000.
South America	1, 3-8, 12, 15	Kuchiishi et al., 2007; Pineda et al., 1996; Rodríguez-Méndez, 2010; Zielinski, 2006.
Australia	1-3, 5, 7, 15	Blackall et al., 1999; Blackall et al., 2002; Eaves and Blackall, 1998.

Table 2. *Actinobacillus pleuropneumoniae* RTX toxins, function and serovar distribution.

RTX toxins	Function	Serovar
ApxI	Strongly hemolytic and cytotoxic for alveolar macrophages and neutrophils	1,5 (a,b), 9,10 and 11
ApxII	Weakly hemolytic and cytotoxic for macrophages and neutrophils	1-9, 11-15
ApxIII	Non-hemolytic but strongly cytotoxic for alveolar macrophages and neutrophils	2-4, 6 and 8
ApxIV	Still not clear, proven to be necessary for full virulence	All serovars

Data was compiled from: Beck et al., 1994; Chiers et al., 2010; Cho and Chae, 2001; Frey et al., 1993; Frey, 1995, 2003); Schaller et al., 1999.

**CHAPTER 3. DETECTION OF *ACTINOBACILLUS PLEUROPNEUMONIAE*
APXIV TOXIN ANTIBODY IN SERUM AND ORAL FLUID SPECIMENS FROM
PIGS INOCULATED UNDER EXPERIMENTAL CONDITIONS**

W Gonzalez, LG Giménez-Lirola, A Holmes, S Lizano, C Goodell,
K Poonsuk, P Sitthicharoenchai, Y Sun, J Zimmerman

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SUMMARY

The prevention and control of *Actinobacillus pleuropneumoniae* (APP) in commercial production settings is based on serological monitoring. ELISAs have been developed to detect specific antibodies against a variety of APP antigens, including long-chain lipopolysaccharides (LPS) and the ApxIV toxin, an RTX exotoxin unique to APP and produced by all serovars. The objective of this study was to describe ApxIV antibody responses in serum and oral fluid from 4 groups of pigs (6 pigs per group) inoculated with APP serovars 1, 5, 7, or 12. Weekly serum samples and daily oral fluid samples were collected from individual pigs for 56 days post inoculation (DPI) and tested by LPS (IgG) and ApxIV ELISAs (IgM, IgA, IgG). All pigs inoculated with APP serovars 1 and 7 were LPS ELISA serum antibody positive from DPI 14 to 56. A transient and weak LPS ELISA antibody response was observed in pigs inoculated with serovar 5 and a single antibody positive pig was observed in serovar 12 at ≥ 35 DPI. ApxIV serum and oral fluid antibody responses in pig inoculated with serovars 1 and 7 reflected the patterns observed for LPS antibody, albeit with a 14 to 21 day delay. This work suggests that ELISAs based on ApxIV antibody detection in oral fluid samples could be effective in population monitoring for APP.

INTRODUCTION

Actinobacillus pleuropneumoniae (APP) is an important bacterial respiratory pathogen of swine causing acute fibrinohemorrhagic and necrotizing pleuropneumonia.⁵¹ In addition, APP is often involved in the porcine respiratory disease complex, a multifactorial infection

resulting from the combination of primary and secondary respiratory pathogens.^{3,4,17} Transmission of APP to swine is commonly ascribed to exposure to contaminated respiratory secretions, and/or aerosols.^{11,56} More recent publications have described environmental contamination of commercial swine production systems with APP, which raises the possibility of indirect routes of transmission.³²

Two APP biotypes have been identified on the basis of the nicotinamide adenine dinucleotide (NAD or V-factor) requirement for growth: biotype I (NAD-dependent) and biotype II (NAD-independent).⁴⁴ APP is further divided into 15 serovars on the basis of capsular polysaccharide antigen composition. Serovars 1-to-12 and 15 belong to biotype 1; serovars 13 and 14 to biotype 2.^{2,43} The degree of virulence among serovars is variable and dependent on several factors, including capsular polysaccharides (CP), lipopolysaccharides (LPS), outer membrane proteins, and the RTX exotoxins, e.g., ApxI, II, III, and IV, produced by all serovars albeit in different combinations.^{14,22,48} Apx toxins are highly immunogenic, i.e., induce antibody production.^{14,51} Importantly, the ApxIV toxin is unique to APP and is produced by all serovars.⁴⁸

The economic impact of APP on the swine industry is a consequence of chronic infections that reduce productivity, i.e., lower feed conversion rates or feed intake; infections that result in acute death, particularly in late finishers; lesions at slaughter; and the costs attributable to prevention, control, and/or elimination of APP.^{32,52} The prevention and control of the APP in commercial production settings is based on the detection of APP infections via serological monitoring. Several APP serum antibody assays have been described and most are available for use in the diagnostic setting, including the complement fixation (CF) test, a variety of enzyme-linked immunosorbent assays (ELISA), and a fluorescent microsphere immunoassay.¹⁵ The CF is a technically complex assay with poor performance attributes (sensitivity and specificity), for which reasons it is used infrequently.¹⁷ In its place, ELISAs have been developed to detect specific antibodies against a variety of APP antigens including, capsular polysaccharide (CP), lipopolysaccharide (LPS), and Apx exotoxins (ApxI, II, III, IV).^{18,23,40}

Although serum is the historic choice for antibody detection and APP surveillance, a variety of antibody-based assays for oral fluid specimens have been reported, e.g., assays for African swine fever virus,³⁹ *Erysipelothrix rhusiopathiae*,¹⁶ influenza A virus,⁴² porcine circovirus type 2,⁴⁵ and porcine reproductive and respiratory syndrome virus.²⁸ The objective of the present study was to describe and compare antibody responses specific for ApxIV in serum and oral fluid from pigs inoculated with APP under experimental conditions using a commercial ApxIV antibody ELISA.

MATERIALS AND METHODS

Experimental design

Four groups of pigs (6 pigs per group) were inoculated with APP serovars 1, 5, 7, or 12. Serum samples were collected weekly and oral fluid samples were collected daily from individual pigs from day post inoculation (DPI) -14 through DPI 56. To confirm infection, serum samples were completely randomized and tested by long-chain lipopolysaccharide (LPS) ELISAs for serovars [(1(9, 11), 5a-5b, 7(4), and 12] at a Service de Diagnostic, University of Montreal, Québec, Canada. Serum and oral fluid samples were tested for ApxIV antibody using a commercial assay.^a Statistical analyses were performed to compare the ApxIV responses among and within serovars over time. The study was approved by the Iowa State University Office for Responsible Research (#8-13-7626-S, #13-I-0019-A).

Animal management and housing

The 24 14-week-old pigs used in the experiment were housed at the Iowa State University Livestock Infectious Disease Isolation Facility, a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. All personnel involved in the experiment had received institutional approval for working with swine and conducting the procedures. Animals were observed a minimum of twice daily throughout the experiment.

Upon arrival, animals were randomly assigned to one of the four APP serovar groups by blindly selecting ear tags from a bag. Each consecutive sequence of six ear tag numbers was assigned to one room (serovar). Animals were weighed upon arrival using a portable

electronic scale^b for the purpose of assessing general health and group uniformity. Pigs were individually housed in pens equipped with nipple drinkers and fed an antibiotic-free commercial diet^c once daily. Hard plastic balls^d were provided for enrichment. Pigs were acclimatized for 2 weeks following placement in the facility, during which time they were trained for oral fluid collection. At 58 DPI, pigs were humanely euthanized by penetrating captive bolt^e followed by exsanguination.³¹

Sample collection

To prevent cross-contamination, four people performed daily feeding and oral fluid sampling chores, i.e., one person for each room (serovar). When it was necessary to move between rooms, e.g., for blood collection, personnel showered and changed coveralls, boots, gloves, masks, and protective eyewear between each room.

Serum samples were collected weekly from DPI -14 through 56 using a single-use blood collection system^f and then centrifuged at 1800 x g for 10 min. Samples were assigned a random number at the time of collection and stored in 2 ml aliquots at -80°C until tested.

Oral fluid samples were collected daily from individual pigs as described elsewhere.^{27,46} In brief, 3-strand, 1/2" (1.27 cm) diameter, 100% cotton rope^g was suspended from a bracket fixed to the side of the pen to allow the pigs to chew on the rope. After 30 min, the chewed (wet) end of the rope was inserted into a plastic bag and severed from the dry portion of the rope. To harvest the fluid, the rope was passed through a wringer^h while in the plastic bag. The fluid that accumulated in the bottom of the bag was then decanted into a 50 ml centrifuge tube.ⁱ Samples were assigned a random number at the time of collection and stored in 4 ml aliquots -80°C until tested.

Inoculum preparation

Four strains of APP were used in this experiment: serovar 1 (ATCC 27088), serovar 5a (ATCC 33377), serovar 7 (ATCC WF83), and serovar 12 (ATCC 9799/84). For propagation, bacteria were cultured on chocolate agar plates^j incubated at 35°C with 5-10% CO₂ for 48 hrs. To check for purity, bacterial isolates were streaked on 5% sheep blood agar

plates.^j Plates were then cross-streaked with *Staphylococcus epidermidis* and incubated at 35°C for 48 hrs.

Prior to preparing the inoculum, serovar identity was confirmed by co-agglutination.³⁶ In brief, bacteria were harvested from chocolate agar plates and mixed with normal saline (~2.5 ml) to a McFarland turbidity of 0.5.³⁸ Thereafter, each bacterial suspension was dispensed onto plastic 3-well blood typing plates^k at a rate of 3 drops per well to which was added one drop of each serovar-specific serotyping reagent to each well.³⁰ After mixing on a mechanical rotator^l for 2 min at a speed of 140 rpm, the serovar was identified by agglutination of the bacterial suspension with a specific serotyping reagent. Agglutination in more than one well was interpreted as an untypeable response.⁵³

To prepare the inoculum, bacteria was harvested from the chocolate agar plate and suspended in normal saline (~2.5 ml) to a McFarland turbidity of 0.5. To estimate the bacterial concentration, 6 ten-fold serial dilutions (10^{-1} to 10^{-6}) were made of the McFarland suspension into sterile saline. 10 μ l of each dilution was pipetted onto a chocolate agar plate and streaked. After 48 hrs of incubation, the numbers of colonies were counted and used to estimate colony forming units (CFU) per ml in the McFarland suspension.^{36, 53}

Inoculation and post-inoculation observations

Prior to inoculation all animals tested negative for APP serum antibody by two commercial ELISAs^{a,m} at the Iowa State University Veterinary Diagnostic Laboratory (Ames, Iowa).

To reduce animal stress and facilitate handling, pigs were sedated immediately prior to inoculation using a solution formulated by reconstituting ⁿ 250 mg of tiletamine, 250 mg of zolazepam with 2.5 ml of xylazine^o (100 mg per ml) and 2.5 ml of ketamine^p (100 mg per ml). An intramuscular dose of 0.05 ml per kg of body weight provided approximately 20 min of sedation. Each room of animals (n = 6) was inoculated with one APP serovar (1, 5, 7 or 12). Animals were inoculated by instilling 2 ml of the inoculum intranasally (IN) and then swabbing the tonsils of the soft palate with a 16" large-tip cotton swab^q saturated with 3 ml of the inoculum. Visualization of the tonsils and the process of tonsil swabbing were

facilitated with the use of an oral speculum. For the first 24 hrs post inoculation, animals were observed for clinical signs and rectal temperatures taken every three hrs by the same individual. Thereafter, clinical observations were taken each morning at the time of oral fluid collection. Any animal exhibiting respiratory distress, emesis, reluctance to stand, and/or a rectal temperature $\geq 41^{\circ}\text{C}$ ($\geq 105^{\circ}\text{F}$) at two consecutive observations was intramuscularly treated with ampicillin^r at a dose of 5 mg per kg.

Serum antibody testing

To confirm infection, serum samples were tested at the Service de Diagnostic University of Montreal (Québec, Canada) with four long-chain lipopolysaccharide (LPS) ELISAs designed to detect capsular serovars [(1(9,11), 5a-5b, 7(4), and 12].

To detect ApxIV IgG antibody, serum samples were tested at Iowa State University using a commercial indirect ELISA.^a The test was performed according to the manufacturer's instructions and a positive response was defined as a sample-to-positive (S/P) ratio ≥ 0.5 .

$$\text{S/P ratio} = \frac{(\text{sample OD} - \text{negative control mean OD})}{(\text{positive control mean OD} - \text{negative control mean OD})}$$

The manufacturer's protocol was also followed for the detection anti-ApxIV IgM and IgA, but the kit IgG secondary antibody conjugate was replaced with goat anti-pig IgM or IgA horseradish peroxidase-labeled conjugate^s diluted 1:2000 and 1:15000, respectively.

Oral fluid antibody testing

Prior to antibody testing, oral fluid samples were processed to remove suspended particulates by adding one ml of sample to a tube containing 100 μl of a lyophilized coagulant formulation (proprietary). Tubes were shaken for one min and centrifuged (4°C) at $1,200 \times g$ for 3 min. The supernatant tested for ApxIV antibody on a commercial serum antibody ApxIV ELISA^a using protocols modified to detect ApxIV IgM, IgA, and IgG in oral fluids. Specifically, ELISA kit serum plate controls were diluted to an OD of 0.1 (negative control)

and 1.3 to 1.5 (positive control) and the ELISA kit IgG secondary antibody conjugate was replaced with goat anti-pig IgM, IgG, or IgA horseradish peroxidase-labeled conjugate^s diluted to 1:2000, 1:3000, or 1:15000, respectively. To perform the test, samples were diluted 1:2 with sample diluent,^a 100 µl of each sample was added to each well, and the plates were incubated at 37°C for 120 min. Plates were then washed 5 times, 100 µl of anti-pig IgG, IgM, or IgA was added to each well, and the plates incubated at 37°C for 60 min. Thereafter, TMB substrate (100 µl) was added to each well and the plates incubated at room temperature for 15 min. Stop solution (100 µl) was added to each well, after which the reaction was read at 450 nm on an ELISA plate reader. The sample-to-positive (S/P) ratios were calculated for each sample using the formula provided by the kit's manufacturer.

Data analysis

Analyses were done using commercial statistical software.^t The results of serological testing were expressed as least square mean values of optical densities and S/Ps by serovar, specimen, and antibody isotype. Figures were created using commercial graphics software.^u Antibody responses within and among serovars were compared using repeated measures ANOVA. If a significant difference was detected, then a *post hoc* test with Bonferoni adjustment was done to compare serovars by DPI.

RESULTS

Clinical observations

As shown in Table 1, the frequency with which clinical signs were observed varied among serovar groups. No clinical signs were observed in pigs inoculated with serovar 12 at any time. Some or all of serovar 5 pigs were reluctant to stand through DPI 2 to 4, but did not exhibit other clinical signs. Pigs inoculated with serovars 1 or 7 exhibited clinical signs in the first 24 hrs post inoculation and through DPI 11, including panting, reluctance to move, vomiting, anorexia and rectal temperature $\geq 105^{\circ}\text{F}$ ($\geq 41^{\circ}\text{C}$). All animals from these two groups were treated with ampicillin from DPI 4 through 11. One pig inoculated with serovar 1 was found dead the morning of DPI 3. Post mortem examination revealed lesions characteristic of APP infection, i.e., tonsils covered in purulent exudate, trachea and bronchi

filled with a foamy exudate, and extensive pleural adhesions. One pig from the serovar 7 group was humanely euthanized on DPI 20 due to lameness unrelated to APP infection.

Sample collection

A total of 202 serum samples were collected from DPI 0 to 56, after which they were completely randomized and tested by 4 LPS ELISAs and the ApxIV ELISA. A total of 1,311 oral fluid samples were collected from DPI -8 to 56, with a mean sample volume of 8.22 ± 5.6 ml (SD) per pig. A subset of oral fluid samples ($n = 1,264$) were completely randomized and tested by ApxIV ELISA.

LPS ELISA serum antibody responses

Serum samples were tested by four LPS ELISAs [(1(9, 11), 5a-5b, 7(4), and 12], with positive results only observed against the serovar to which animals were exposed. The LPS ELISA serum antibody response varied significantly among serovars (Figure 1, Table 2). Based on the manufacturer's recommended cutoff of $OD \geq 0.40$, all but 2 samples from pigs exposed to serovars 1 ($n = 5$) and 7 ($n = 6$) were positive from DPI 14 through DPI 56. In serovar 5, 4 of 6 pigs were positive at DPIs 14, 21, 28; 2 pigs were positive at DPI 35; and one pig was positive at DPI 42 and later. In serovar 12, all pigs were negative, except for one pig that tested positive on DPIs 35, 42, and 49.

Within serovars, comparisons showed that all serovar 1 OD responses at $DPI \geq 7$ were higher than DPI 0 OD responses ($p < 0.0001$). For serovars 5 and 7, ODs at DPI 14 and later were significantly higher than DPI 0 ($p < 0.0001$). For serovar 12, pairwise comparisons found no difference in mean OD responses when compared to DPI 0 except for DPI 49 ($p = 0.038$). Between serovars, the magnitude of LPS OD responses differed significantly over time ($p < 0.0001$). Comparisons by DPI detected no difference between serovar 1 and 7 OD responses. At DPIs 14 through 56, serovar 1 and 7 responses were significantly higher than serovars 5 and 12 ($p < 0.0001$). At DPIs 14 through 42, serovar 5 OD responses were higher than serovar 12 ($p < 0.0001$).

ApxIV ELISA serum antibody responses (Table 3)

ApxIV IgG ELISA ApxIV serum antibody (IgG) ELISA S/P responses varied among serovars. Based on the manufacturer's recommended cutoff ($S/P \geq 0.50$), 3 of 5 pigs in serovar 1 and 5 of 5 pigs in serovar 7 became positive over the course of the study. No pigs in serovars 5 or 12 met the manufacturer's criterion for positivity.

Within serovars, all serovar 1 and 7 IgG ELISA S/P responses at DPI 21 and later were significantly higher than their DPI 0 S/P responses ($p < 0.0001$). In serovars 5 and 12, pairwise comparisons found no difference in S/P responses by time when compared to DPI 0.

A comparison among serovars showed that the serovar 1 IgG S/P response was higher than serovars 5 and 12 at DPI 28 and later ($p < 0.0033$). The serovar 7 IgG S/P response was higher than the serovar 1 response at DPI 21 ($p = 0.0090$) and 56 ($p = 0.0301$) and higher than serovars 5 and 12 at DPI 14 and later ($p < 0.0040$). No difference was detected in the serovar 5 and 12 responses over time.

ApxIV IgM ELISA Within serovars, serovar 1 and 7 IgM S/P responses were higher than DPI 0 responses at DPI 14 ($p < 0.0001$). In serovars 5 and 12, no difference was found in IgM responses at any time point when compared to DPI 0. Between serovars, the serovar 7 IgM S/P response was higher than serovars 1, 5, and 12 at DPIs 14 and 21 ($p < 0.0020$). Serovar 1 IgM S/P responses differed from serovar 12 at DPI 14 ($p = 0.0163$) and DPI 42 ($p = 0.0264$).

ApxIV IgA ELISA Within serovars, comparisons detected IgA S/P responses higher than DPI 0 at DPI 56 ($p = 0.0373$) in pigs exposed to serovar 1. Likewise, detectable differences were found on IgA responses in pigs exposed to serovars 5 at DPI 35 ($p = 0.0485$) and DPI 42 ($p = 0.0015$). No detectable differences were found on serovars 7 and 12 when compared with DPI 0. Comparisons among serovars found no detectable difference in the magnitude of IgA S/P responses over time.

ApxIV ELISA oral fluid antibody responses (Table 4)

ApxIV IgG ELISA ApxIV oral fluid antibody (IgG) ELISA S/P responses varied among serovars. In serovar 1, the IgG S/P responses were higher than DPI 0 on DPI 15 and later ($p = 0.0096$). In serovar 5, IgG S/P responses higher than DPI 0 were detected at DPI 41 ($p = 0.0267$). In serovar 7, S/P responses greater than DPI 0 were detected at DPI 14 and later ($p < 0.0313$). In serovar 12, no difference in IgG response was found at any sampling point when compared to DPI 0.

Comparisons between serovars showed that the magnitude of the IgG S/P antibody response differed between serovars 1 and 7 at DPI 22 ($p = 0.0229$), DPI 35 ($p = 0.0013$) and later. The serovar 1 IgG S/P response was higher than serovars 5 and 12 at DPI 16 and later ($p < 0.0403$). Likewise the serovar 7 response was higher than serovars 5 and 12 at DPI 15 and later ($p < 0.0361$). No difference was detected in serovar 5 and 12 responses over time.

ApxIV IgM ELISA In serovar 1, IgM S/P responses were higher than DPI 0 at DPI 29 ($p = 0.0157$). In serovar 5, IgM S/P responses were higher than DPI 0 at DPI 3 through 11 ($p = 0.0355$), and at DPI 45 ($p = 0.0163$) through 56 ($p = 0.0161$). In serovar 7, IgM responses were higher than DPI 0 at DPI 13 ($p = 0.0261$), DPI 14 ($p = 0.0157$) and DPI 15 ($p < 0.0001$). In serovar 12, no difference in IgM response was found at any sampling point when compared to DPI 0.

Comparisons between serovars showed that the serovar 7 IgM S/P response was higher than serovars 1, 5 and 12 at DPIs 11 through 15 ($p < 0.0488$) and then higher than serovar 1 at DPI 43 ($p = 0.0143$) and DPI 46 ($p < 0.0001$). The serovar 1 IgM S/P response was higher than serovar 12 at DPI 29 ($p = 0.0139$). No difference was detected in serovar 5 and 12 responses over time when compared to each other.

ApxIV IgA ELISA In serovar 1, IgA S/P responses were higher than DPI 0 at DPI 44 ($p = 0.0482$), 52 ($p = 0.0371$), and DPI 55 ($p < 0.0001$). In serovar 7, IgA responses were higher from DPI 0 at DPIs 10 and 11 ($p = 0.0307$), then on DPIs 14-19 ($p < 0.0218$), DPI 30-56 ($p < 0.0001$). No detectable differences were found on serovars 5 and 12 over time when

compared with DPI 0. Comparisons between serovars detected no differences in the IgA S/P responses among serovars 1, 5, and 12 at any sampling point. The serovar 7 IgA S/P response was higher than serovars 1, 5, and 12 at DPI 23 and later ($p < 0.0001$).

DISCUSSION

The goal of this study was to describe ApxIV-specific antibody responses in serum and oral fluid from pigs inoculated with APP under experimental conditions. ApxIV is only produced by APP, is produced by all serovars, and is highly immunogenic, i.e., produces detectable levels of serum antibodies in pigs.^{8,12,21} For these reasons, ApxIV antibody offers the potential to monitor APP infections in the field. Detection of ApxIV antibody has not been previously described in swine oral fluids, although oral fluid has been shown to be a suitable specimen for the detection of antibodies against a variety of pathogens, as reviewed elsewhere.^{27,46}

The outcome of inoculating pigs with APP under experimental conditions depends on the route and dose.^{1,49} Several routes of APP inoculation have been described, including IN,^{25,37} endotracheal,¹ endobrochial,²⁶ direct application of the inoculum to the tonsils of the soft palate,⁷ and aerosol.¹⁹ Reproducing APP infections under experimental conditions is complicated by the fact that lower challenge doses may not result in infection.^{55,56} On the other hand, higher doses may cause severe clinical signs and/or acute death.⁴⁷

As distinct from experimental inoculation, APP infections in the field are believed to occur by direct contact between pigs or via aerosol exposure.^{11,17} However, recent research has shown that the environment (drinking water, feed, soil) is also heavily seeded with APP,³² suggesting that field infections may involve repeated low-dose exposures.⁵⁶ Thus, differences among experimental results may reflect variability in the exposure route and dose under experimental conditions^{24,25,55,56} and differences between experimental results and field observations may result from the fact that experimental exposures do not reflect the mode of APP transmission (dose, route, frequency) in the field.²⁰

In the present study, animals were inoculated both IN and by direct application of the inoculum to the tonsils of the soft palate. The production of clinical signs was marked and prolonged in the two groups (serovars 1 and 7) exposed to APP at a concentration of 1×10^6 CFU per ml and mild or absent in groups exposed to APP at a concentration of 1×10^5 CFU per ml (serovars 5 and 12).

The kinetics of the LPS-specific serum antibody response have been described. After aerosol exposure to serovar 1, LPS-specific IgM was reported at 14 DPI, IgA at 14 to 21 DPI, and IgG at 21 DPI.⁵ Following IN exposure to serovar 9, LPS-specific IgM was detected at 7 DPI and both IgA and IgG at 14 DPI.²⁹ In the current study, the LPS serum antibody response in serovars 1 and 7 was in agreement with previous reports. That is, an LPS-specific IgG response was detected as early as 7 DPI, with all animals seropositive by DPI 14. Thereafter, all animals remained seropositive through DPI 56. Thus, the LPS serum antibody ELISA response was compatible with "productive" APP infection in animals inoculated with serovars 1 and 7. In contrast, a weak and transient LPS ELISA response was observed in animals inoculated with serovar 5. In serovar 12, a single animal was LPS ELISA positive on DPI 35, 42, and 49. These results are compatible with previous studies reporting variable success at infecting pigs with APP under experimental conditions,^{9,10} even at very high exposure doses.^{41,54}

There are relatively few studies on ApxIV serum antibody ontogeny and these are based on the detection of ApxIV-specific IgG. ApxIV IgG was reported to be detected at 7 to 28 DPI in pigs inoculated with serovars 1, 5b, 6, 7, 10, or 15 via aerosol exposure.¹² In other study, seroconversion to ApxIV was reported at 3 to 6 weeks post inoculation in pigs exposed to serovars 1, 7, or 15 by either IN or endotracheal routes.¹³ In the current study, using the ApxIV ELISA S/P results from DPI 0 as the basis for comparison, a significant increase ($p < 0.05$) in ApxIV antibody was detected in serum at 14 DPI (IgM) and 21 DPI (IgG) in animals inoculated with serovars 1 and 7. This pattern was consistent with previous reports,^{12,13} and also followed the pattern observed for the LPS ELISA response in these animals.

The results of this study suggested that the S/P cutoff of the commercial serum ApxIV IgG ELISA is too high. That is, using the manufacturer's recommended cutoff ($S/P \geq 0.50$), "seroconversion" was delayed in some animals inoculated with serovars 1 or 7 up to DPI 49. However, a limitation of the current study was the small number of animals. As a consequence, it was not possible to conduct a valid statistical analysis of test performance, e.g., receiver operating characteristic analysis, to re-calculate the ELISA cutoff and associated test sensitivity and specificity.

In a previous report, APP IgA was detected in stimulated oral fluid samples collected 14 DPI after the IN inoculation of pigs with serovar 2, but there are no previous reports of ApxIV antibody in swine oral fluid.³³ Using the oral fluid ApxIV ELISA S/P results from DPI 0 as the basis for comparison, a significant increase ($p < 0.05$) in ApxIV IgG S/P ratios was detected at ≥ 21 DPI in pigs inoculated with serovar 1, with no detectable increase in IgM or IgA. In serovar 7, significantly higher IgG S/P ratios were observed at ≥ 14 DPI. In this group, a significant increase ($p < 0.05$) in the ApxIV IgM response was observed at 14 DPI and in the IgA response at DPI 21 and then at ≥ 35 DPI.

Respiratory diseases are among the most important issues in swine health worldwide. Among other determinants, the increasing intensification of pork production and the development of large, technified pig farms have created conditions suitable for the spread of agents associated with swine respiratory diseases.³⁵ The development of tools for monitoring respiratory infections is a key component in the control of these diseases. Previous studies have demonstrated the efficiency of swine oral fluids for the detection of antibody against ASFV,³⁹ influenza A virus,⁴² porcine circovirus type 2,⁴⁵ and porcine reproductive and respiratory syndrome virus²⁸ and others. Based on the data presented herein, it can be concluded that assays for detecting APP ApxIV antibody in oral fluids could also be developed for monitoring APP infections in commercial herds.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declare the following potential conflicts of interest with respect to the research

authorship, and/or publication of this article: authors S. Lizano and C. Goodell are employed by IDEXX Laboratories, Inc. The remaining authors declare no conflicting interests with respect to their authorship or the publication of this article.

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SOURCES AND MANUFACTURERS

- a. ApxIV Ab ELISA, IDEXX Laboratories, Inc., Westbrook, ME.
- b. Pelouze ® 4040-88 Electronic weighing scale, Rubbermaid Inc., Huntersville, NC.
- c. Heartland CO-OP, West Des Moines, IA.
- d. Otto Environmental, Greenfield, WI.
- e. Accles and Shelvoke, Ltd., Sutton Coldfield, UK.
- f. Kendall Company, Mansfield, MA.
- g. Grainer Industrial Supply, DSM, IA.
- h. WC38K, Dyna-Jet Products, Overland Park, KS.
- i. Corning Inc., Corning, NY.
- j. Remel, Inc., Lenexa, KS.
- k. Fisherbrand™, Pittsburgh, PA.
- l. TekTator® V, American Hospital Supply Corp, Evanston, IL.
- m. Swinecheck® Mix-APP, Biovet, Inc., St. Hyacinthe, Quebec, Canada.
- n. Telazol®, Fort Dodge Animal Health, Fort Dodge, IA.
- o. Lloyd Laboratories, Shenandoah, IA.
- p. Fort Dodge Animal Health, Fort Dodge, IA.
- q. Birchwood Laboratories, Inc., Eden Prairie, MN.
- r. Polyflex®, Boehringer Ingelheim, Vetmedica, St Joseph, MO.
- s. Bethyl, Montgomery, TX.
- t. SAS® version 6.1.7601, Microsoft Corporation, USA.
- u. SigmaPlot® version 12.5, Systat Software, Inc.

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Table 1. Clinical observations following inoculation with *Actinobacillus pleuropneumoniae*

		Day Post Inoculation ^a							
OBSERVATION		0 ^b	1	2	3	4	5	6	7
Serovar 1	Reluctant to stand	6	6	6	5	4	4	1	1
	Respiratory distress	6	6	6	5	5	5	5	5
	Emesis	5	5	5	5	5	5	5	5
	Rectal temperature ≥ 41 C	1	• ^c	•	•	•	•	•	•
	Anorexia	6	•	6	5	5	2	2	0
	Oral Fluid collection (≥ 1 ml)	4	0	0	1	2	3	3	3
Serovar 5	Reluctant to stand	6	6	6	2	2	•	•	•
	Respiratory distress	•	•	•	•	•	•	•	•
	Emesis	•	•	•	•	•	•	•	•
	Rectal temperature ≥ 41 C	•	•	•	•	•	•	•	•
	Anorexia	•	•	•	•	•	•	•	•
	Oral Fluid collection (≥ 1 ml)	6	3	3	5	5	4	5	4
Serovar 7	Reluctant to stand	6	6	6	6	6	6	6	6
	Respiratory distress	6	6	6	6	6	6	6	6
	Emesis	6	6	6	2	2	•	•	•
	Rectal temperature ≥ 41 C	1	•	•	•	•	•	•	•
	Anorexia	6	6	6	6	6	6	6	6
	Oral Fluid collection (≥ 1 ml)	5	0	0	0	0	3	5	0
Serovar 12	Reluctant to stand	•	•	•	•	•	•	•	•
	Respiratory distress	•	•	•	•	•	•	•	•
	Emesis	•	•	•	•	•	•	•	•
	Rectal temperature ≥ 41 C	•	•	•	•	•	•	•	•
	Anorexia	•	•	•	•	•	•	•	•
	Oral Fluid collection (≥ 1 ml)	6	5	6	6	5	6	6	5

^aDenominator is 6 pigs in all groups at all DPIs, except serovar 1 (one pig died on DPI 2).

^bFirst 24 hours following inoculation.

^cSymbol (•) indicates not observed in any pigs in the group.

Table 2. *Actinobacillus pleuropneumoniae* qualitative serum antibody responses by serovar over time based on serogroup capsular LPS ELISAs^a

APP Inoculum	LPS ELISA	Pig status	Day Post Inoculation								
			0	7	14	21	28	35	42	49	56
Serovar 1 ATCC 27088	1-9-10-11	positive	1	3	5	4	4	5	5	5	5
		suspect	0	1	0	0	0	0	0	0	0
		negative	5	1	0	1	1	0	0	0	0
		no. pigs	6	5	5	5	5	5	5	5	5
Serovar 5 ATCC 33377	5a-5b	positive	0	0	4	4	4	2	1	1	1
		suspect	0	0	1	1	1	3	4	2	1
		negative	6	6	1	1	1	1	1	3	4
		no. pigs	6	6	6	6	6	6	6	6	6
Serovar 7 ATCC WF83	4-7	positive	0	1	6	6	5	5	5	4	5
		suspect	0	4	0	0	0	0	0	0	0
		negative	6	1	0	0	0	0	0	0	0
		no. pigs	6	6	6	6	5	5	5	5 ^b	5
Serovar 12 ATCC 9799/84	12	positive	0	0	0	0	0	1	1	1	0
		suspect	0	0	0	0	0	0	0	0	0
		negative	6	6	6	6	6	5	5	5	5
		no. pigs	6	6	6	6	6	6	6	6	6 ^b

^aAPP LPS ELISA interpretation based on optical density (OD): negative OD < 0.30, suspect OD 0.30 - 0.39, positive OD ≥ 0.40.

^bMissing data on one pig.

Table 3. *Actinobacillus pleuropneumoniae* ApxIV serum antibody ELISA (S/P) response over time

APP Inoculum	Isotype	Day Post Inoculation								
		0	7	14	21	28	35	42	49	56
Serovar 1 ATCC 27088	IgM	0.02	0.23	0.43^a	0.29^a	0.27^a	0.35^a	0.47^a	0.41^a	0.29^a
	IgA	0.03	0.08	0.12	0.14	0.19	0.18	0.11	0.34	0.37^a
	IgG	-0.03	-0.00	0.09	0.12^a	0.29^a	0.49^a	0.47^a	0.58^a	0.53^a
Serovar 5 ATCC 33377	IgM	0.12	0.34	0.29	0.23	0.17	0.19	0.32	0.17	0.31
	IgA	0.05	0.11	0.16	0.17	0.19	0.35^a	0.53^a	0.31	0.29
	IgG	-0.02	-0.00	-0.01	-0.00	-0.00	-0.00	0.01	0.00	0.02
Serovar 7 ATCC WF83	IgM	0.19	0.20	1.32^a	0.73^a	0.44^a	0.38^a	0.35^a	0.33^a	0.25^a
	IgA	-0.01	0.00	0.03	0.07	0.14	0.11	0.16	0.08	0.05
	IgG	-0.02	-0.00	0.24	0.36^a	0.38^a	0.46^a	0.44^a	0.63^a	0.73^a
Serovar 12 ATCC 9799/84	IgM	0.01	0.02	0.02	0.05	0.18	0.23	0.09	0.14	0.17
	IgA	0.19	0.27	0.44	0.39	0.29	0.08	0.27	0.42	0.18
	IgG	-0.2	-0.02	-0.02	-0.02	0.02	0.02	-0.02	-0.02	-0.03

^aWithin serovars, S/P responses were significantly higher than DPI 0 responses (ANOVA, $p < 0.05$)

Table 4. *Actinobacillus pleuropneumoniae* ApxIV oral fluid antibody ELISA (S/P) response over time

APP Inoculum	Isotype	Day Post Inoculation								
		0	7	14	21	28	35	42	49	56
Serovar 1 ATCC 27088	IgM	0.05	0.07	0.06	0.15	0.05	0.04	0.04	0.02	0.02
	IgA	0.07	0.16	0.10	0.12	0.11	0.10	0.13	0.12	0.19
	IgG	0.00	0.01	-0.00	0.17^a	0.23^a	0.24^a	0.42^a	0.25^a	0.21^a
Serovar 5 ATCC 33377	IgM	0.07	0.03	0.06	0.06	0.13	0.05	0.05	0.03	0.02
	IgA	0.03	0.04	0.05	0.05	0.10	0.08	0.20	0.06	0.08
	IgG	-0.01	-0.01	-0.00	0.00	0.00	-0.00	0.00	-0.01	-0.00
Serovar 7 ATCC WF83	IgM	0.09	0.13	0.39^a	0.11	0.13	0.17	0.05	0.06	0.07
	IgA	0.00	0.23	0.25^a	0.11	0.30	0.56^a	0.46^a	0.39^a	0.47^a
	IgG	-0.03	0.06	0.17^a	0.23^a	0.29^a	0.60^a	0.43^a	0.41^a	0.47^a
Serovar 12 ATCC 9799/84	IgM	0.04	0.11	0.06	0.06	0.05	0.07	0.05	0.02	0.02
	IgA	0.04	0.10	0.08	0.08	0.09	0.12	0.08	0.05	0.07
	IgG	0.01	0.01	0.01	0.01	0.00	0.01	0.00	-0.00	-0.00

^aWithin serovars, S/P responses were significantly higher than DPI 0 responses (ANOVA, $p < 0.05$)

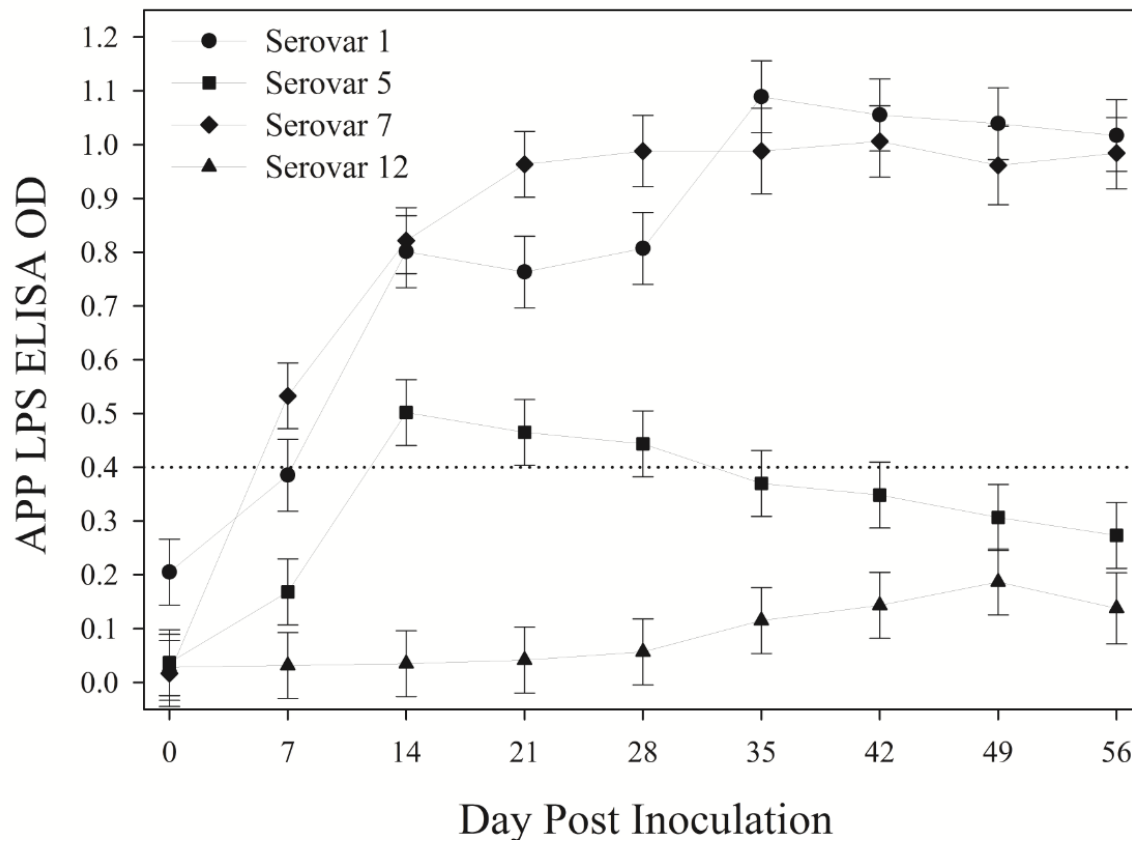


Figure 1. *Actinobacillus pleuropneumoniae* quantitative serum antibody responses^a (least square means) by serovar over time based on serogroup LPS ELISAs.

^a APP LPS ELISA interpretation is based on optical density (OD): negative OD < 0.30, suspect OD 0.30 - 0.39, positive OD \geq 0.40.

CHAPTER 4. GENERAL CONCLUSIONS

Actinobacillus pleuropneumoniae (APP), the causative agent of porcine pleuropneumonia, is found throughout the world and may be a significant health issue wherever swine are produced commercially (Sebunya and Saunders, 1983). APP infections are often acute and rapidly fatal, but animals can also develop chronic and/or subclinical infections which are important because they can serve to seed future outbreaks (Gottschalk, 2012).

Interest in APP arose with the recognition of the considerable economic losses attributable to the disease, including the costs of identifying infected herds (Gottschalk, 2012; Losinger, 2005). Several preventive measures have been described and, with some limitations, are considered effective (Gottschalk, 2012; MacInnes et al., 1988; Sebunya and Saunders, 1983).

Overall, APP has been (and remains) an “under-studied” pathogen, i.e., funding for research has been minimal. This may be justified by the perception that foreign, transboundary, or emerging viral pathogens pose a greater threat (directly or indirectly) to the industry.

Whether this will still be true as the use of antimicrobials in veterinary medicine become more restricted remains to be seen. Even so, APP is a clinical and economic problem in many parts of the world and in some North American swine production systems. If control or elimination of APP is to be achieved in such circumstances, specific information and/or technology is needed. In particular, a review of the literature shows that a better and more complete understanding of the epidemiology and ecology of APP is needed.

As the industry continues to consolidate, the development of tools for monitoring respiratory infections will be a key component in the control of these diseases. Previous studies have demonstrated the efficiency of swine oral fluids for the detection of antibody against ASFV (Mur et al., 2013), influenza A virus (Panyasing et al., 2013), porcine circovirus type 2 (Prickett et al., 2008), and porcine reproductive and respiratory syndrome virus (Kittawornrat et al., 2012) and others. Based on the data presented herein, it can be concluded that assays for detecting APP ApxIV antibody in oral fluids could also be developed for monitoring APP infections in commercial herds and should be a priority for future research.

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