UNIVERSITY OF MINNESOTA

EVALUATION OF *ACTINOBACILLUS PLEUROPNEUMONIAE* DIAGNOSTIC TESTS USING SAMPLES FROM EXPERIMENTALLY INFECTED PIGS.

By

Giordana Souza Costa
Dr. Simone Oliveira, Advisor (Chair)
Department of Veterinary Medicine of Population

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(ABSTRACT)

The early detection of pigs subclinically infected with *A. pleuropneumoniae* is a critical step in avoiding the introduction of this agent into naïve populations. Negative herds are usually monitored for the presence of *A. pleuropneumoniae* using serology. At least 3 different tests are available for detection of antibodies against *A. pleuropneumoniae* and results obtained with each of these tests often disagree. Unexpected or discordant serological results are usually further investigated by direct detection of *A. pleuropneumoniae* in the tonsils by isolation and/or PCR. At this time, there is no information available on how these tests compare regarding detection of *A. pleuropneumoniae* infection or which combination of tests provides a better definition of true infection status. In order to address these questions, we have evaluated the performance of different serological tests, isolation, and PCR for detection of *A. pleuropneumoniae* infection using samples from pigs experimentally infected with 7 clinically relevant serotypes. This thesis offers a comprehensive assessment of the diagnostic tools available for characterization of *A. pleuropneumoniae* subclinical infection.

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LITERATURE REVIEW

Etiology

Actinobacillus pleuropneumoniae is a gram-negative, encapsulated, facultative anaerobic, non-spore forming, nonmotile, pleomorphic coccobacillus (Kilian et al., 1984). It belongs to the family *Pasteurellaceae*, which also includes the genera *Haemophilus, Actinobacillus* and *Pasteurella*. The first field isolates of *A. pleuropneumoniae* were reported in the early 1960's in Great Britain, California, and Argentina (Matthew et al., 1961; Shope, 1964). At that time the isolates were respectively classified as *Haemophilus parainfluenzae*, *Haemophilus parahaemolyticu*s, and *Haemophilus pleuropneumoniae*. Additional biochemical characterization of the ability of the organism to ferment certain sugars established that it represented a distinct bacterial species. In 1983, DNA hybridization studies by Pohl et al. indicated that there was no significant homology between *H. pleuropneumoniae* and *H. influenzae*. However, the homology between *H. pleuropneumoniae* and *Actinobacillus lignieresii* was significant. These findings resulted in a proposal to classify this organism under the *Actinobacillus* genus and consequently change the nomenclature from *H. pleuropneumoniae* to *Actinobacillus pleuropneumoniae* (Pohl et al., 1983).

Actinobacillus pleuropneumoniae grows on blood agar supplemented with NAD or V factor. This factor can also be supplemented by a nurse *Staphyloccocus sp*. culture

with *A. pleuropneumoniae* typically growing close to the *Staphylococcus sp.* streak (satellitism). *Actinobacillus pleuropneumoniae* forms 0.5-1mm hemolytic colonies after 24 hours of incubation on blood agar (Straw et al., 1999). It produces a positive CAMP-like reaction when co-cultured with beta-hemolytic *S. aureus* related to the production of three cytolysins: ApxI, ApxII and ApxIII (Frey et al., 1994; Jansen et al., 1995). Table 1.1 shows the biochemical characteristics of *A. pleuropneumoniae* used for bacterial identification.

Actinobacillus pleuropneumoniae can be classified into two biovars based on their NAD requirement. Biotype I strains are NAD dependent, whereas biotype II strains can synthesize NAD in the presence of specific pyridine nucleotides or their precursors (Bossé et al., 2002). Presently, there are 15 known serotypes of *A. pleuropneumoniae*, with serotypes 1 to 12 and 15 classified as biotype I and serotypes 13 and 14 classified as biotype II (Blackall et al., 2002). Atypical strains of serotypes 2, 4, 7 or 9 can also behave as biotype II. These strains have been exclusively reported in Europe (Gottschalk, 2007).

Table 1.1 – *Actinobacillus pleuropneumoniae* biochemical characterization.

TEST	OUTCOME
V-factor dependency	+
Hemolysis	+
CAMP	+
Catalase	-
Urease	+
Indole production	-
Lysine decarboxylase	-
Ornithine decarboxylase	-
ONPG	+
Nitrate reduction	+
Acid form	
Glucose	+
Mannitol	+
Inositol	-
Sorbitol	-
Rhamnose	-
Saccharose	+
L-Arabinose	-
Galactose	+
Esculin	-

Source: Moller and Kilian (1990)

Epidemiology

Actinobacillus pleuropneumoniae is a strict colonizer of the porcine upper respiratory tract. It is usually isolated from tonsils and less frequently from the nasal cavity of carrier pigs (Kume et al., 1984). All age groups are susceptible to development of clinical disease caused by this pathogen with growing pigs being most frequently affected group. Factors such as movement, crowding, and adverse climate conditions play an important role on the onset and severity of the disease (Nicolet, 1992). Pigs

surviving the infection develop a serotype-specific protective immunity and may become subclinical carriers of the pathogen (MacInnes et al, 1988).

Transmission of *A. pleuropneumoniae* can occur through direct contact between sows and piglets (vertical transmission) or between infected carriers and naïve pigs (horizontal transmission) (Nicolet, 1992; Marsteller et al., 1999; Dubreil et al., 2000). Vertical transmission of this pathogen occurs at a later timing compared to most colonizers, which explains the success of segregated early weaning practices in eliminating *A. pleuropneumoniae* from the offspring of infected sows (Marsteller et al., 1999).

A major question regarding *A. pleuropneumoniae* transmission is whether this pathogen can be spread through the air, and if so, how far it can travel. Airborne transmission has been reproduced experimentally using serotype 1 over 1 meter distance (Tomorremorell et al., 1997) and serotype 9 over 2.5 meters (Jobert et al., 2000). The presence of *A. pleuropneumoniae* in air samples has also been documented by PCR in conventional swine herds under experimental conditions (Nielsen et al., 2000). Some studies have reported the potential airborne transmission of *A. pleuropneumoniae* serotypes 1 and 2 over short distances (0.1 to 0.25 miles) using antimicrobial sensitivity patterns as epidemiological markers (Desrosiers et al., 1998; Fussing et al., 1998). Area spread of *A. pleuropneumoniae* over distances varying from 400 to 500 meters associated with dominant winds has also been proposed using similar methodology (Desrosiers et al., 1998).

Most epidemiological studies involving *A. pleuropneumoniae* are based on serotyping information. The geographical distribution, variety of *A. pleuropneumoniae* serotypes, and the virulence of specific serotypes differs considerably among countries (Blackall et al., 2002, Desrosiers et al., 1984). Serotypes 1, 5, and 7 have been reported as the most prevalent and clinically relevant in the United States (Masteller et al, 1999), serotypes 2 and 9 as the most relevant in Europe (Dubreiul et al., 2000), and the newly described serotype 15 as the most predominant and virulent serotype affecting Australian herds (Blackall et al., 1996). The presence and clinical relevance of *A. pleuropneumoniae* serotype 15 has also recently been reported in Canada, USA, Mexico, Brazil and Argentina (Gottschalk, 2007). Prevalence and dominant *A. pleuropneumoniae* serotypes in different countries are shown in Table 1.2.

Table 1.2 – Geographic distribution of *Actinobacillus pleuropneumoniae* serotypes by geographic location

COUNTRY	PREVALENT SEROTYPES	DOMINANT SEROTYPES	REFERENCES
Argentina	1, 2, 3, 5, 12	1	Vena et al., 1997, 1988
Australia	1, 2, 3, 7, 12, 15	1	Eaves and Blackall et al., 1988; Gottschalk, 2007
Belgium	2, 3, 6, 7, 8, 9, 11	3	Hommez et al., 1988,1990
Brazil	1, 3, 4, 5, 7, 9	5, 3	Piffer et al., 1997
Canada	1, 2, 3, 5, 6, 7, 8, 10, 12, 15	5, 7, 1, 12	Rosendal et al., 1981b; Mittal et al., 1982, 1992, 1998; Gottschalk, 2007
Chile	1, 5	1, 5	Olivares and Morgado, 1988
Croatia	2, 7, 8, 9	2, 9	Habrun et al., 1998
Czech Republic	1, 2, 4, 5, 7, 12	9, 2, 11	Skollova and Gois, 1987; Kucerova et al, 2005
Denmark	1, 2, 3, 5, 6, 7, 8, 10, 11, 12	2	Nielsen, 1982, 1987
France	2, 3, 7, 8, 9	9	M. Kobisch, personal communication 1990
Germany	2, 3, 4, 5, 6, 7, 9, 10	9, 2, 7	Schimmel and Hass, 1983; Muller et. al., 1986 Kielstein and Wuthe, 1988
Hungary	1, 2, 3, 5, 6, 7, 9, 10, 11, 12	3, 2, 7	Fodor et al., 1989; Molnar, 1990, 1992
Italy	1, 2, 3, 4, 5, 7	5	Dubreil et al., 2000
Ireland	3	3	Power et al., 1983
Japan	1, 2, 3, 5, 6, 7, 8, 9, 12, 15	2, 1, 5	Chan et al., 1978; Kume et al., 1986; Fukuyasu et al, 1991; Koyoma et al, 2007
Korea	2, 3, 5, 7	5, 2	Yeh, 1990
Mexico	1, 2, 3, 4, 5, 6, 7, 8, 9	1, 8	Ciprian et al., 1988; Diazi et al., 1988; Ontiveros-Corpus et al., 1995
Netherlands	1, 2, 3, 5, 7, 8, 9, 11	2, 9, 11	Kamp et al., 1987
Norway	2	2	Falk et al., 1991
Poland	1, 2, 5, 9	1, 9	Molenda, 1988; Tarasiuk et al., 1991
Spain	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12	4, 7, 2	Ferri et al., 1990; Gutierrez et al., 1995
Sweden	2, 3, 4	2	Gunnarsson, 1978
Switzerland	2, 3, 7, 9	2	Nicolet, 1988, 1992
Taiwan	1, 2, 3, 5	1, 5	Hung et al., 1991; Chang and Chang, 1994
Thailand	11, 5, 3, 1, 6, 9	11, 5	Assavacheep et al., 2003
UK	1, 2, 3, 5, 6, 7, 8, 10	2, 3, 8	Hunter et al., 1983; Brandreth and Smith, 1985; McDowell and Ball, 1994
USA	1, 3, 5, 7, 8, 9, 15	1, 5	Schultz et al., 1983; Hoffman et al., 1985; Rapp et al., 1985; Fales et al., 1989; Gottschalk, 2007
Venezuela	1, 7, 4, 2, 3, 6	1	Pinda et al., 1996

Expanded from Dubreuil et al., 2000.

Clinical signs

An outbreak of *A. pleuropneumoniae* can result in varying degrees of clinical disease: peracute, acute, sub-acute, and chronic (Dubreuil et al., 2000). The clinical manifestations of *A. pleuropneumoniae* infection vary with age, background immunity, environmental conditions, and level of exposure to the agent (Nielsen, 1982; Shope et al., 1964). The course of the disease differs from animal to animal, depending on the extent of the lung lesions and the time of initiation of therapy. All stages of disease may develop within an affected group (Marsteller et al., 1999).

In the peracute form, pigs become suddenly ill and may reach body temperatures of up to 106.7 F (41, 5˚C) (Nicolet, 1992). They may also exhibit increased respiratory rate, coughing/sneezing, anorexia, ataxia, vomiting, diarrhea, and severe respiratory distress (Bossé et al., 2002). These symptoms are usually followed by circulatory failure and cyanosis (Nicolet, 1992). In the absence of treatment, the disease can progress very rapidly and death can occur within a few hours. In the terminal phase, animals remain in sitting posture showing a severe dyspnea, mouth breathing, and decreased rectal temperature. Hemorrhagic froth arising from pulmonary edema may be discharged from the nose or mouth just prior to death (Taylor, 1999). Sudden death with absence of clinical signs may also occur (Sebunya et al., 1983).

In the acute form, several pigs in the same or different pens are usually affected. Body temperatures also rise to 105-106 F (40.5-41˚C), the skin may be reddened, the animals may look depressed, are reluctant to rise, to eat and drink (Pijpers et al., 1990).

Respiratory difficulties become evident and circulatory failure may occur. The acute form of the disease may lead to death or recovery (Nicolet, 1992). If the pig is able to survive the first four days of the outbreak, it is likely to complete the production cycle (Lo et al., 1998).

A chronic form of the disease often persists in those animals that survive the acute phase (Nielsen, 1985). Chronic infections are characterized by persistent coughing with fibrous pleuritis being a frequent sequelae. Poor growth and increased susceptibility to secondary infections have also been reported (Gottschalk, 2007; Dubreuil et al., 2000). Most cases are asymptomatic with evidence of chronic infection being only observed at slaughter (Shope, 1964).

Lesions

Lesions associated with *A. pleuropneumoniae* infection are mainly concentrated in the lungs and are characterized by bilateral pneumonia with involvement of the cardiac, apical, and/or diaphragmatic lobes. Pneumonic lesions are often focal and well demarcated (Nicolet, 1992; Taylor, 1999). On acutely affected pigs, lung lesions are dark pink to plum red and blood stained froth may be observed in the trachea. Fibrinous adhesions between lungs, thoracic wall, diaphragm, and pericardium are also frequent findings (Rogers et al., 1990). Tracheobronchial and mesenteric lymph nodes may become distended as a result of infiltration of inflammatory cells and fibrin deposition

(Bossé et al., 2002). Microscopically, lung lesions are characterized by infiltration of polymorphonuclear cells (neutrophils), fibrin exudation with deposits in blood and lymph vessels, hemorrhage, and necrosis (Sebunya et al., 1983).

Animals that survive infection may have complete resolution of lesions, but frequently they retain focal necrotic areas due in part to the influx of host immune cells and the release of neutrophil contents that include oxygen radicals and/or well encapsulated abscesses with overlying areas of fibrinous connective tissue (Bossé et al., 2002). Chronic lung lesions can be difficult to differentiate from lesions caused by other pathogens (Fenwick et al., 1994). These lung lesions are mottled red to yellow and are less extensive with more pronounced fibrosis (Rogers et al., 1990). Chronically infected animals may also harbor *A. pleuropneumoniae* in tonsillar crypts. Although epithelial cell damage, edema, and PMN infiltration of the tonsils has been described following direct application of large numbers of *A. pleuropneumoniae,* there has been no description of tonsillar pathology in chronically infected animals (Bossé et al., 2002). Chronic microscopic lesions are more prominent in the lungs and pleura and are characterized by macrophage infiltration, marked fibrosis around areas of necrosis, and fibrous pleuritis (Taylor, 1999).

Pathogenesis and virulence

The pathogenesis of *A. pleuropneumoniae* is multifactorial and involves coordinated action of several different virulence attributes of the bacterium including surface polysaccharides (Bandara et al., 2003; Jacques, 2004; Ramjeet et al., 2005), Apx toxins (Frey, 1995; Liu et al., 2009), iron uptake systems (Haesebrouck et al., 1997; Jacques, 2004), biofilm formation (Kaplan et al., 2005), components of anaerobic metabolism (Baltes et al., 2003; Baltes et al., 2005; Buettner et al., 2008; Buettner et al., 2009; Jacobsen et al., 2005), and outer membrane proteins (Chung et al., 2007).

All 15 reported serotypes are able to cause disease but they vary in virulence (Jacobsen et al., 1996). The observed differences in virulence within and between serotypes are due to capsular structure, LPS composition, and hemolysins secreted (Perry et al., 1990; Ward et al., 1997; Taylor, 1999). The immune status of the animals and the general condition of the herd may also determine the virulence potential of a specific strain (Gottschalk, 2007). Following is a brief review of three well characterized virulence factors for *A. pleuropneumoniae* and their roles in pathogenesis.

Capsular polysaccharides (CPS)

The capsular polysaccharides *(CPS)* of *A. pleuropneumoniae* are serotype-specific structures responsible for the antigenic characterization of the 15 different serotypes recognized so far (Jessing et al., 2008). The capsule is also the primary component that protects bacterium from host defenses such as phagocytosis and complement-mediated

killing and it is required for virulence (Dubreuil et al., 2000). These capsules are negatively charged and are composed of oligosaccharide units, techoic acid polymers joined by phosphate diester bonds, or oligosaccharide polymers joined by phosphate bonds (Perry et al., 1990). When plated on clear medium, the capsule of *A. pleuropneumoniae* can be visualized as sparkling shade surrounding the colonies (Inzana, 1991). The composition and structure of CPS determine the variation in virulence among strains (Dubreuil et al., 2000; Bandara et al., 2003). In general, serotypes possessing larger and more adherent capsule tend to be more virulent than serotypes with less capsule (Jensen et al., 1986). Evaluation of genetically identical strains suggested that the amount and the type of CPS produced or its mechanism of expression also determine virulence of *A. pleuropneumoniae* in pigs (Bandara et al., 2003). The *A. pleuropneumoniae* capsule has a very limited immunogenic capacity (Fenwick et al., 1986; Inzana et al., 1987a). Purified capsule fails to activate the complement cascade and does not demonstrate toxic activity (Ward et al., 1994; Fenwick et al., 1986). No clinical symptoms or lung lesions were found when purified capsule was administered endobronchially to pigs (Fenwick et al., 1986). Although purified *A. pleuropneumoniae* CPS do not induce clinical illness or pulmonary lesions in pigs, the capsule is essential for *A. pleuropneumoniae* virulence *in vivo* since it allows the bacterium to resist the antibacterial environment produced by the host's immune system (Dubreiul et al., 2000).

Lipopolysaccharides (LPS)

Lipopolysaccharides (LPS) are essential structural components of the outer-membrane of gram-negative bacteria. These structures are involved in *A. pleuropneumoniae* adherence to porcine respiratory tract cells and are considered important factors in pathogenesis and virulence. LPS are mainly formed by three distinct regions: the lipid A, a core region of common sugars, and an O-polysaccharide side chain (Dubreuil et al., 2000). Each serotype has a particular composition and structure of the LPS O-side chain (Fenwick et al., 1986; Byrd et al., 1989). However, immunological cross-reactivity may be observed in some serotypes with the similar O-side chain composition (Fenwick et al., 1986). Cross-reactive serotypes include 1, 9, 11, serotypes 3, 6 and 8, and serotypes 4 and 7 (Dubreiul et al., 2000). Purified LPS has the potential to cause damage to lung tissue; however it does not cause typical hemorrhagic and necrotic lung lesions by itself (Udeze et al., 1987).

Toxins

Four toxins belonging to the RTX (repeats in toxin) family of hemolytic/cytotoxic proteins have been described among the different strains of *A. pleuropneumoniae* and they are referred to as ApxI, ApxII, ApxIII, and ApxIV (Kamp et al., 1991; Frey et al., 1993; Jansen et al., 1995; Schaller et al., 1999; Bandara et al., 2003). Toxin profiles are inherent to *A. pleuropneumoniae* serotypes, as shown in Table 1.3 (Frey, 1995). The characteristic lesions induced by *A. pleuropneumoniae* infection are directly associated with the release of these toxins during infection (Bertram, 1986).

ApxI is a strongly hemolytic and cytotoxic protein of 105-110 kDa, expressed *in vivo* and *in vitro,* and secreted by the most virulent serotypes, including 1, 5, 9, 10 and 11 (Kamp et al., 1991; Frey et al., 1990, Schaller et al., 1999). ApxII is a weakly hemolytic and moderately cytotoxic protein of 103-105 kDa produced by all serotypes except for serotype 10 (Schaller et al., 1999). The ApxII toxin alone seems to confer moderate virulence (Frey, 1995). ApxIII is a protein of 120 kDa secreted by serotypes 2, 3, 4, 6 and 8. It differs from the other two toxins because it does not possess any hemolytic activity; however it is strongly cytotoxic (Schaller et al., 1999).

ApxIV is the most recently described toxin being for *A. pleuropneumoniae*. This toxin is weakly hemolytic and is produced only *in vivo* by all serotypes (Schaller et al., 1999). Although the role of the ApxIV on *A. pleuropneumoniae* pathogenesis still needs further clarification, studies have confirmed that deletion of the *apxIVA* gene led to attenuation of a virulent strain, suggesting that ApxIV is likely essential for the expression of the full virulence of the bacterium. ApxIV is expressed only *in vivo* and is highly specific for *A. pleuropneumoniae*. This makes this toxin a great candidate for differentiation between infected and vaccinated animals (Liu et al., 2009).

Table 1.3 – Toxin profiles for *Actinobacillus pleuropneumoniae* reference serotypes.

SEROTYPE	APX I	APX II	APX III	APX IV
1	+	+	-	+
2	-	+	+	+
3	-	+	+	+
4	-	+	+	+
5	+	+	-	+
6	-	+	+	+
7	-	+	-	+
8	-	+	+	+
9	+	+	-	+
10	+	-	-	+
11	+	+	-	+
12	-	+	-	+
13	-	+	-	+
14	-	+	-	+
15	-	+	-	+

[+] Presence of the toxin production; [-] Absence of the toxin production. (Schaller et al., 1999).

Immunity

Natural infection by *A. pleuropneumoniae* results in the development of protective immunity (Nielsen et al., 1979; Nielsen et al., 1984; Inzana, 1991). Disease expression is dose dependent and lower exposure results in seroconversion without clinical disease, whereas a slightly higher level of exposure results in fatal infection (Sebunya et al., 1983). Pigs that survive acute infection frequently become subclinical carriers of the pathogen (Lo et al., 1998; Dreyfus et al., 2004).

Maternal immunity is transmitted from sows to piglets and may last up to 12 weeks (Cruijesen et al., 1995; Chiers et al., 2002). Pigs with high maternal antibody

titers can still be colonized with *A. pleuropneumoniae*, which confirms that maternal immunity does not interfere with colonization. Although it does not prevent colonization, maternal immunity does protect against the development of lung lesions (Chiers et al., 2002).

Protective immunity against *A. pleuropneumoniae* can also be achieved by vaccination. Several vaccines have been developed and used to prevent mortality caused by this agent. Similar to maternal immunity, vaccination does not prevent colonization or development of carrier state (Fewinch 1986, Chiers 2002). Both subunit and chemically inactivated whole cell bacterins have been used to prevent clinical signs and mortality caused by *A. pleuropneumoniae.* A subunit vaccine containing Apx toxins I, II, and III, and a transferrin binding protein is able to provide cross-protective immunity among serotypes, whereas killed whole cell products are usually serotype-specific. (Rossi-Campos et al., 1992; Cruijsen et al., 1995; Haesebrouck et al., 1997).

Reports for timing of seroconversion following infection vary greatly in the literature. This variation is in part due to differences in experimental designs, challenge strains, challenge doses, sources of pigs, and tests used to detect seroconversion (Bossé et al., 1992; Klausen et al., 2002; Gottschalk et al., 1994 and 2003; Dreyfus et al., 2004). Table 1.4 summarizes the earliest time of seroconversion following infection by different serovars.

Table 1.4 – Timing for seroconversion to *A. pleuropneumoniae* following experimental infection.

SEROTYPE (CHALLENGE)	ELISA ANTIGEN	EARLIEST DETECTION	REFERENCE
1	CPS	3-4 wpi*	Bossé et al (1992)
5	LPS and CPS	2 wpi	Klausen et al (2002)
7	CPS	3-4 wpi	Bossé et al (1992)
9	LC-LPS	2 wpi	Gottschalk et al (2003)
1, 5, 6, 7, 9, 10, 15	ApxIV	3wpi average	Dreyfus et al (2004)

*wpi = weeks post infection

Prevention and Control

Antibiotic treatment, vaccination, management and husbandry practices are considered key steps to control and eradicate *Actinobacillus pleuropneumoniae* (Chiers et al., 2002). Isolation of naïve populations is essential to maintain negative status. New animals brought into a naïve herd must be purchased from known disease-free or specific pathogen free herds and be quarantined and tested prior to introduction (Rosendal et al., 1983, MacInnes et al., 1988; Straw et al., 1999). Serology is widely used to monitor exposure to *A. pleuropneumoniae* and to detect subclinically infected pigs prior to pig movement (Gottschalk 1997). Carrier status can be confirmed by the direct detection of *A. pleuropneumoniae* in the tonsils by isolation and PCR (Klein et al., 2003).

An all-in all-out policy should also be implemented (Straw et al., 1999). A study, which compared the use of all-in-all-out management to continuous flow management, demonstrated that all-in all-out management was better overall for health and growth of

pigs and could reduce the prevalence and severity of pneumonia in pigs of all ages (Ice et al., 1999). Clark et al (2000) reported that weaning at 14 days of age followed by age-segregated rearing was sufficient to eliminate transfer of *A. pleuropneumoniae* to progeny of infected sows. In addition, the immunization of herds to prevent and control infection with *A. pleuropneumoniae* has been successfully used to reduce mortality. However, vaccination does not prevent colonization, development of chronic lesions, or development of carrier animals (Fenwick et al., 1986; Goethe et al., 2001; Chiers et al., 2002).

Diagnostics

Diagnosis of *A. pleuropneumoniae* infection can be accomplished by direct detection of the agent in the tonsils or lungs by isolation and/or PCR, or indirect detection of exposure to the agent by serology. This section will review the application, advantages, and limitations of each of these techniques.

Isolation

Actinobacillus pleuropneumoniae can be easily recovered from lungs of animals with peracute or acute disease. Isolation from carriers, on the other hand, is often unsuccessful due to the contamination of tonsil samples by the commensal flora present at the upper respiratory tract (Sidibé 1993).

Several methods have been developed as an attempt to improve *A. pleuropneumoniae* isolation from contaminated samples. Gilbride et al. (1983) isolated *A. pleuropneumoniae* from 5% of the samples when culturing the cut surface of tonsils of 80 slaughter pigs on TSA agar plates supplemented with lincomycin, bacitracin, crystal violet, and NAD. Moller et al. (1993) was able to isolate A. pleuropneumoniae from 22% of 303 pigs tested at slaughter using chocolate agar supplemented with 300 µg/ml bacitracin. Sidibé et al. (1993) found that PPLO-agar supplemented with 1 µg/ml lincomycin, 2µg/ml crystal violet, 156µg/ml bacitracin and 0.0001% NAD was more effective for isolation of *A. pleuropneumoniae* from tonsils compared to isolation using blood agar or TSA supplemented with the same components.

Another way to identify the presence of *Actinobacillus pleuropneumoniae* is by using magnetic beads coated with antibodies against specific serotypes of *A. pleuropneumoniae*, also known as immunomagnetic separation technique (IMS) (Gagné et al., 1998). After the antibody-coated beads captured the bacteria, contaminating flora is washed away and nearly pure cultures of *A. pleuropneumoniae* can be obtained. The sensitivity of the IMS technique is 1000-fold higher than that of direct culture (Gottschalk, 1999-AASV).

Serological assays

Complement fixation (CF)

The complement fixation (CF) test is based on the use of complement to detect antigen-antibody complexes (Mittal et al., 1984). For many years, this test was considered the standard method for detection of anti-*A. pleuropneumoniae* antibodies (Hoffman, 1989). Although extensively used in the past, CF has several disadvantages that have restricted its use nowadays. More specifically, results are highly variable and are not reproducible with some pigs moving from positive to negative status in a short period of time (Hoffman et al., 1989). CF testing is also laborious and requires intensive standardization. It is time consuming, expensive, and relatively insensitive when compared to other assays such as the enzyme-linked immunosorbent assay (ELISA), for example (Lo et al., 1998).

Enzyme Linked Immunosorbant Assay (ELISA)

The use of an enzyme-linked immunosorbent assay (ELISA) for detection of antibodies against *A. pleuropneumoniae* was first proposed by Nicolet et al. (1981) as an alternative to the CF test. ELISA tests utilized to detect exposure to *A. pleuropneumoniae* can be based on a variety of antigens, such as capsule (Inzana et al., 1987b; Bossé et al., 1990; Bossé et al., 1992), LPS (Fenwick et al., 1986; Gottschalk et al., 1994b), or toxin (Dreyfus et al., 2004). ELISA is known to be more sensitive and specific compared to traditional CF testing (Inzana et al., 2001). Although highly sensitive, ELISA tests may

fail to detect antibodies in carrier pigs in the absence of clinical signs or lesions (Fittipaldi et al., 2003).

Currently, there are three tests commercially available for detection of antibodies against *A. pleuropneumoniae:* Swinecheck® APP ELISA offered by Biovet, Multi-APP, offered by the University of Montreal Veterinary Diagnostic Laboratory, and the Chekit APPApxIV® ELISA, offered by IDEXX. Swinecheck® APP ELISA tests are serotype-specific and detect antibodies against serotypes (1-9-11), 2, (3-6-8-15), (4-7), 5, 10, 12 and 13 (Gottschalk et al., 1994 and 1997; Radacovici et al., 1994 and 1995). This test targets the long chain LPS of *A. pleuropneumoniae* and is unable to differentiate cross-reactive serotypes. The Multi-APP ELISA also detects antibodies against the long chain LPS of *A. pleuropneumoniae*. It contains a mixture of antigens from all *A. pleuropneumoniae* serotypes and has been used as a screening. The Chekit APPApxIV® ELISA test detects antibodies against the Apx IV toxin, which is expressed by all *A. pleuropneumoniae* serotypes during infection (*in vivo*). This test is able to detect seroconversion in animals that have had acute and chronic infection; however, it may not be able to detect carriers without previous infection since expression of Apx IV may only happen after lung infection (Schaller et al., 1999, Dreyfus et al., 2004).

Polymerase Chain Reaction

PCR is widely used to identify healthy carrier pigs in subclinically infected populations. In the absence of clinical signs, the tonsils of carriers are not like to harbor

large number of the organism (Fittipaldi et al., 2003), thus PCR is a valuable tool for identification of carrier pigs.

Several PCR techniques have been described for detection of *A. pleuropneumoniae* in clinical samples. These tests target different genes, such as *oml*A (Savoye et al., 2000), *dsb*E-like (Chiers et al., 2001), *aro*A (Hernanz et al., 1999), *cps-cpx* (Lo et al., 1998), and *apx*IV (Schaller et al., 2001). A comparison of these tests using samples from naturally and experimentally infected pigs demonstrated that the *apx*IV-based PCR described by Schaller (2001) was highly specific and sensitive, detecting a minimum of 10^2CFU/g of tonsil (Fittipaldi et al., 2003b). This test can be used to screen animals for *A. pleuropneumoniae* colonization and was considered to be the best alternative when culture step is avoided and maximal analytical sensitivity is desired (Fittipaldi et al., 2003).

Serotyping

Indirect Hemagglutination test (IHA)

The indirect hemagglutination (IHA) test is based on the detection of serotype-specific antigens using rabbit polyclonal antiserum. Serotype-specific antigens are mainly of capsular origin (Nielsen, 1982). This test is widely used for *A. pleuropneumoniae* serotyping. IHA is able to identify most *A. pleuropneumoniae* serotypes; however, it occasionally fails to differentiate serotypes with similar polysaccharides, such as 1-9 and 3-6-8 (Blackwell et al., 1999; Nielsen et al., 1984). IHA can separate serotype 5 into two

subgroups: 5a and 5b (Nielsen, 1986). The clinical relevance of this subgrouping needs to be further evaluated.

Serotyping by PCR

The serotype of an *A. pleuropneumoniae* isolate may be defined using two different PCR methods: a serotype-specific PCR, which detects the capsular gene of a given serotype (Fittipaldi et al., 2003) and a toxin profiling PCR, which define serotypes based on detection of toxin genes (Frey, 1995; Rayamajhi et al., 2005). Serotype-specific PCR tests are available for serotypes 1, 2, 3, 5, 6, 7, 8, and 12. (Lo et al., 1998; Jessing et al., 2003; Schuchert et al., 2004; Hüssy et al., 2004; Zhou et al., 2008). There are no tests for serotypes 4, 9, 10, 11, 13, 14, 15. Toxin profiles are inherent to a specific serotype, and detection of ApxI, Apx II, Apx III, and Apx IV by PCR can be used to identify the serotype of any isolate (Table 1.5). Toxin profiling by PCR can group *A. pleuropneumoniae* isolates into the following groups: serotypes 1, 2-8-15, 3, 4, 5, 6, 7, 9-11, 10, 12-13, and 14 (Jessing et al., 2003). Differentiation among isolates identified as 2-8-15, 9-11, and 12-13 can usually be achieved by IHA.

Table 1.5 – Polymerase chain reaction amplification of *apx*I, *apx*II, *apx*III, and *apx*IV genes from the 15 serotypes of *Actinobacillus pleuropneumoniae.*

SERO TYPES	*apx*IA 723bp	*apx*IB 811bp	*apx*II 965bp	*apx*III 396bp	*apx*IV 1600bp	*apx*IV 2000bp	*apx*IV 2400bp	*apx*IV 2800bp
1	+	+	+				+	
2		+	+	+				+
3			+	+			+	
4		+	+	+	+			
5a	+	+	+					+
5b	+	+	+					+
6		+	+	+		+		
7		+	+					+
8		+	+	+				+
9	+	+	+		+			
10	+	+						+
11	+	+	+		+			
12		+	+				+	
13		+	+				+	
14	+	+					+	
15		+	+	+				+

(Rayamajhi et al, 2005).

CHAPTER 2

Evaluation of *Actinobacillus pleuropneumoniae* diagnostic tests using samples of experimentally infected pigs

OVERVIEW

New serological tests have recently been introduced for *A. pleuropneumoniae* (APP) diagnosis. No information is currently available on how these tests compare regarding the detection of antibodies from subclinically infected pigs. To answer this question, 80 pigs were randomly assigned to experimental groups infected with *A. pleuropneumoniae* serotypes 1, 3, 5, 7, 10, 12, 15 and a non-inoculated control group. Blood samples and oropharyngeal swabs were collected prior to infection and for 7 consecutive weeks thereafter. Serum samples were tested using the Swinecheck® APP ELISA and the Multi-APP ELISA (University of Montreal). All pigs were euthanized at 49 days post-inoculation. Tonsil and lung samples were cultured for isolation and tested by PCR. The Multi-APP ELISA detected seroconversion one week earlier than the Swinecheck ® APP ELISA with the earliest seroconversion detected at 1 week post-infection (serotype 10) and the latest at 3 weeks post-infection (serotype 1). Seroconversion at day 49 was serotype-dependent and varied from 4 (44%) positives detected in the serotype 10 group to 9 positives (100%) detected in the serotype 15 group. Thirty-one pigs were serologically positive for *A. pleuropneumoniae* at 49 days post-infection and only 15 still carried *A. pleuropneumoniae* on the tonsils based on PCR results. No cross-reactions were observed when serum samples were cross-tested using the Swinecheck® APP ELISA. *A. pleuropneumoniae* was successfully isolated from the lung of 2 pigs that developed pleuropneumonia, but was not isolated from tonsils due to heavy contamination by the resident flora. This study offers a comprehensive evaluation of the diagnostic tools currently available for detection of *A. pleuropneumoniae* subclinical infection.

Introduction

Actinobacillus pleuropneumoniae (APP) is the causative agent of the swine pleuropneumonia, a highly contagious disease responsible for significant economic losses worldwide (Jessing et al., 2003; Gottschalk et al., 2003; Dreyfus et al., 2004). This pathogen is absent from most U.S. swine herds. Consequently, monitoring for negative status is very important to avoid the introduction of positive animals into naïve populations (Gottschalk et al., 2006). The early identification of subclinically infected animals is very important to prevent the spread of this pathogen (Rosendal et al., 1982; Gottschalk, 1999-AASV; Taylor, 1999). A wide range of diagnostic tools can be used to diagnose *A. pleuropneumoniae* infection and most are effective for confirming infection when lesions are present. The detection of carriers, on the other hand, is usually not straightforward due to limitations in the sensitivity of the current tests.

Bacterial isolation and serotype-specific ELISA tests were for many years the techniques of choice to diagnose and monitor *A. pleuropneumoniae* infections in naïve and positive swine herds. Given that isolating *A. pleuropneumoniae* from subclinically infected pigs is frequently unsuccessful, serological testing has become the cornerstone of identifying carriers (Montaraz et al., 1996). A number of different serological tests have been used to detect *A. pleuropneumoniae* antibodies, including enzyme labeled immunosorbent assays (ELISA) and the complement fixation (CF) test. ELISA tests reportedly have the best sensitivity and specificity, with those based on long-chain LPS antigens being the most used (Klein et al., 2003). The complement fixation test (CF),

which was once considered the reference serological test for *A. pleuropneumoniae*, is now rarely used as it lacks sensitivity and is relatively complex to perform (Gottschalk et al., 2006). In addition, serum titers in naturally infected animals are highly variable with some infected animals never manifesting significant titers and other animals moving from positive to negative status in a short period of time (Klausen et al., 2002).

Currently, there are at least three different ELISA tests commercially available to diagnose *A. pleuropneumoniae* infections, namely the serotype-specific Swinecheck® APP ELISA offered by Biovet, the Multi-APP ELISA offered at the University of Montreal, and the Chekit APPApxIV® ELISA offered by IDEXX. The serotype-specific Swinecheck® APP and the Multi-APP ELISA tests target similar antigens, specifically the long chain lipopolysaccharide (LC-LPS) of *A. pleuropneumoniae*. The Chekit APP-ApxIV® ELISA targets a species-specific toxin produced by *A. pleuropneumoniae* during infection (Schaller et al., 1999). Results obtained using LC-LPS and ApxIV-based tests frequently disagree due to differences in the target antigens, test sensitivity, and specificity. When serological tests fail to define herd status, clinical history, bacterial isolation, and PCR testing are used to confirm or rule out actual infection.

Considering the limitations of routine isolation of *A. pleuropneumoniae* from tonsils, detection by PCR has become the technique of choice to troubleshoot serological results. Several PCR techniques have been described for detection of *A. pleuropneumoniae* in clinical samples. These tests target different genes, such as *oml*A, *dsb*E-like, *aro*A, *cps-cpx*, and *apx*IV. A comparison of these tests using samples from

naturally and experimentally infected pigs demonstrated that the *apx*IV-based PCR described by Schaller (2001) was highly specific and sensitive, detecting a minimum of 10^2CFU/g of tonsil (Fittipaldi et al., 2003b).

At this time, there is no information available on how the variety of diagnostic tests described above compare regarding detection of *A. pleuropneumoniae* subclinical infection or which combination of tests provides an accurate definition of true infection status. Although many studies have evaluated some of the tests mentioned separately, none have compared all of them in a single experiment. The studies currently reported in the literature evaluate these test under different experimental designs, utilizing different *A. pleuropneumoniae* serotypes, different infection outcomes (clinical or subclinical), and duration of screening. These variables are important confounders that impair the development of a standard protocol for *A. pleuropneumoniae* diagnostics. The objective of this study was to evaluate the capacity of different diagnostics tests to detect *A. pleuropneumoniae* subclinical infection using samples from pigs experimentally infected with 7 clinically relevant *A. pleuropneumoniae* serotypes.

Materials & Methods

Animals

This experiment was approved by the University of Minnesota Institutional Animal Care and Use Committee (IACUC) (protocol # 0806A37381). Eighty six-week

old pigs obtained from a source free of *A. pleuropneumoniae* based on clinical history, tonsil PCR, and serological testing (Chekit APP-ApxIV® ELISA) were transferred to the isolation unit at University of Minnesota. Pigs were randomly divided into 8 different rooms with 10 pigs per room. Each group was inoculated with a different *A. pleuropneumoniae* serotype and one group remained as the negative control (Table 2.1). Pigs were acclimatized for one week pre-infection and housed for seven weeks post-inoculation. All pigs were euthanized at 49 days post infection when post-mortem examinations were conducted to evaluate the presence and severity of *A. pleuropneumoniae* lesions.

Table 2.1 – Strain identification and group assignment for *Actinobacillus pleuropneumoniae* serotypes used for experimental infection.

GROUP	APP SEROTYPE	STRAIN
1	1	4074
2	3	1421
3	5	K17
4	7	WF83
5	10	22009
6	12	1096
7	15	HS143
8	Negative control	-

Bacterial strains

The references strains used to experimentally infect naïve pigs with *A. pleuropneumoniae* serotypes 1 (4074), 3 (1421), 5 (K17), and 7 (WF83) were obtained at the American Type Culture Collection (ATCC). Reference strains for serotypes 10

(22009), 12 (1096) and 15 (HS143) were kindly provided by Dr. Pat Blackall (Blackall et al., 2002) (Table 1). These serotypes were chosen based on their prevalence in U.S. swine herds as identified by querying the Minnesota Veterinary Diagnostic Laboratory (MVDL) database between 2002 and 2008. Although serotype 10 was not isolated from any clinical cases at the MVDL in the past 6 years, it was included in this study due to the frequent detection of antibodies by the serotype-specific Swinecheck® APP ELISA test in pigs from herds with no clinical history of *A. pleuropneumoniae* infection.

Inoculum and challenge

The growth curve for each challenge strain was determined prior to the preparation of the inoculum. *A. pleuropneumoniae* strains were plated on chocolate agar and incubated at 37ºC in 5% CO_2 for 24 hours. Two full loops of each plate were transferred to 50mL of PPLO broth with added NAD and this suspension was incubated for 18 hours. Following incubation, *A. pleuropneumoniae* concentrations were determined using a spectrophotometer (optical density at 280nm) and bacterial counts were confirmed by plate dilutions. One milliliter of each tube was transferred to new tubes with additional 50 mL of PPLO. The bacterial load for each suspension was determined at 2, 4, 6 and 8 hours of incubation using a spectrophotometer and confirmed by plate dilutions. Colony counting for each serotype was performed at 24 and 48 hours. The growth curve was determined for each serotype and the exponential growth phase identified. Challenge inoculum cultures were harvested at 6 hours of growth in PPLO and the concentration was estimated using a spectrophotometer and adjusted to 106 CFU/mL

using PBS. All inoculums were plated for colony counting prior to inoculation. All pigs were inoculated intranasally with 1mL of the respective inoculum in each nostril within 2 hours of its preparation.

Clinical signs

Animals were observed for three consecutive days post inoculation for the development of clinical signs of *A. pleuropneumoniae* infection and weekly thereafter. Rectal temperatures were collected for three consecutive days after inoculation to monitor the course of infection. Pigs that were found to be lethargic with evident respiratory signs and rectal temperature above 105° F were treated with ceftiofur sodium at a dosage of 3 mg/kg body weight administered intramuscularly for two consecutive days.

Blood samples

Blood samples were collected prior to the inoculation (day 0) and weekly thereafter for seven consecutive weeks. Five milliliters of blood were collected from each pig by venipuncture of the jugular vein. Samples were left in the refrigerator (4 ºC) overnight and centrifuged the next day to separate the serum. All serum samples were divided into 1.5 ml aliquots, placed into identified boxes, and frozen at -80°C until assayed. Serum samples were tested weekly using the serotype-specific Swinecheck® APP ELISA tests at the MVDL following the manufacturer instructions and with the Multi-APP ELISA test at the University of Montreal following the standard operational

procedure from this institution. Cross-reactivity among the serotypes that successfully infected the pigs was evaluated by testing serum samples collected at 49 days post-infection using all serotype-specific Swinecheck® APP ELISA tests.

Oropharyngeal swabs

Oropharyngeal swab samples were taken prior to inoculation (day 0) and weekly thereafter for seven consecutive weeks. Two swabs were taken every week from each pig, one for bacterial isolation and one for DNA extraction. The oropharyngeal swabs collected for bacterial isolation were inoculated in 5% sheep blood agar plates with a nurse *Staphylococcus epidermidis* streak. All plates were incubated at 37º C in a 5% CO2 atmosphere for 48 hours. Plates were checked for the presence of *A. pleuropneumoniae* suspect colonies at 24 and 48 hours. Suspect colonies (hemolytic with satellitism to the *S. epidermidis* nurse streak) were re-isolated onto blood agar and incubated in similar conditions for 24 additional hours. DNA from pure cultures was extracted and tested by PCR described by Schaller (2001) to confirm the identity of the isolate. Oropharyngeal swabs collected for DNA extraction were placed into identified centrifuge tubes (1.5mL), suspended in 300µL of PBS (phosphate buffer saline), and vortexed for 1 minute. Swab tips were removed from the tubes and the remaining suspensions were centrifuged at 13,000 rpm for 3 min. The supernatant was discarded and the remaining pellet was re-suspended in 200µL of Prepman Ultra (Applied Biosystems, Foster City, CA). The resuspended content was vortexed for an additional 15 seconds and then boiled for 20 min using a heat block. After boiling, the suspension was again centrifuged at 13,000 rpm

for 3 min to separate the DNA from cell debris. A total of 50µL of DNA from the supernatant was mixed with 50µL of DNA-Rnase-free water for use as a template for the PCR reactions.

Necropsy

All pigs were examined for the presence of gross lesions. Oropharyngeal swabs, whole tonsils, and lung swabs were collected for *A. pleuropneumoniae* isolation and PCR. Whole tonsils were removed using sterile scissors and forceps for each pig. Fragments of 1 cm^2 were obtained from each tonsil for DNA extraction. Each fragment was placed on a sterile plastic bag with 300 µl of PBS, homogenized using a 'stomacher' and 100 µl of the tissue homogenate was transferred to a 1.5 ml tube for DNA extraction. DNA extraction from tissue homogenate was done using the Qiagen DNeasy Blood & Tissue Kit following the manufacturer's instructions. DNA extraction from swabs and bacterial isolations were performed as described previously (oropharyngeal swabs section).

Statistical analysis

The time between inoculation to seroconversion of each pig was evaluated by the Long-rank (Matel-cox) method using Graphpad Prism 5® software. Survival analysis was performed comparing the differences in the detection of positive pigs by both serological tests (Swinecheck® APP and Multi APP ELISA). In order to identify which test provided the earliest precise detection of positive pigs, suspect pigs identified using

the Swinecheck® APP ELISA were considered negative. The Multi-APP ELISA test only generates positive or negative results.

Results

Experimental infection

The serotypes of all reference strains were confirmed by toxin profiling (Rayamajhi et al., 2005) and indirect hemagglutination (Mittal et al., 1992) prior to inoculation. The expected inoculum dose of 10^6 CFU/mL was confirmed for all serotypes by plate counting. Successful infection was confirmed in groups inoculated with serotypes 1, 3, 7, 10, and 15 based on serology and tonsil PCR results at day 49 post-infection. Pigs in groups inoculated with serotypes 5 and 12 did not become infected and remained serologically and PCR negative for the duration of the study.

Clinical signs and necropsy findings

All pigs were clinically examined for three consecutive days post-inoculation and weekly thereafter. Only pigs from groups infected with serotypes 10 and 15 showed clinical signs characterized by respiratory distress and lethargy within 24 hours post-inoculation. Elevated rectal temperatures (average greater than 105° F) were only observed in the group of pigs infected with serotype 10 (Table 2.2). One pig from this

group died despite two consecutive days of treatment with ceftiofur and presented lung hemorrhage and necrosis.

One pig from the group infected with serotype 15 was euthanized at 3 weeks post-infection due to a leg injury unrelated to the experimental infection. At necropsy, lesions characteristic of *A. pleuropneumoniae* (necrosis and hemorrhage in the diaphragmatic lobes of the lungs) were also observed in the lungs of this animal. At 49 days post-infection, mild focal fibrous adhesions between the surface of the lungs and the parietal pleura were observed in pigs infected with serotypes 5 (n=1), 7 (n=4), 10 (n=4), and 15 (n=2). No lesions were observed in pigs inoculated with serotypes 1, 3, and 12 (Table 2.2).

Table 2.2 – Average (μ) and standard deviation (*SD*) rectal temperatures for each experimental group at 24 and 48 hrs post inoculation; and number of pigs with *post mortem* lesions per group.

SEROTYPE	**24h.**	**48h.**	**#LESIONS***
	μ ± *SD* TºC	**μ ± *SD* TºC**	
1	104.01 ± 0.73	103.81 ± 1.33	0
3	104 ± 0.45	103.44 ± 0.70	0
5	104.13 ± 0.81	104.01 ± 0.73	1
7	104.11 ± 0.60	104 ± 0.52	4
10	106.52 ± 1.25	106.11 ± 1.26	4
12	103.84 ± 0.62	103.81 ± 1.33	1
15	104.01 ± 0.73	103.92 ± 0.94	2
Control group	104.61 ± 0.45	103.8 ± 0.60	0

*Focal fibrous adherences in the lungs

Bacterial isolation

A. pleuropneumoniae was not isolated from oropharyngeal swabs collected weekly throughout the experiment or from clinical samples collected at necropsy on day 49 post infection. *A. pleuropneumoniae* was only isolated from pleuropneumonia lesions observed in one pig inoculated with serotype 10 and one pig inoculated with serotype 15 at 3 and 21 days post infection, respectively.

PCR results

The swabs used in this study (BD BBL CultureSwab™) to collect tonsils samples contained a gel-based media in the bottom which interfered with the efficiency of the PCR. Results were inconsistent and were not reproducible due to this interference. Reliable PCR results were only obtained at 49 days post-infection when tonsil fragments were collected and tested. Table 2.3 shows the number of positive animals per serotype group obtained by PCR on tonsil's fragments collected at 49 days post-infection. Infection was confirmed in groups infected with serotypes 1, 3, 7, 10, and 15. All pigs inoculated with serotypes 5 and 12 and all pigs from the non-inoculated control groups were negative for the presence of *A. pleuropneumoniae* in the tonsils by PCR.

Table 2.3 – Serological and PCR results for all experimental groups

		SEROTYPE-SPECIFIC ELISA		MULTI-APP ELISA		PCR
Serotype	N	Earliest + detection (wk)	+/suspect/- at 49 d.p.i	Earliest + detection (wk)	+/- at 49 d.p.i	(+/-)
1	10	5**	4/1/5	3	5/5	1
3	10	3	1/4/5	2	5/5	3
5	10	-	0/0/10	-	0/0	0
7	9*	2	8/0/1	2	8/1	5
10	9*	2	1/3/5	1	4/5	3
12	10	-	0/0/10	-	0/0	0
15	9*	2	5/2/2	2	9/0	3
control	9*	-	0/0/9	-	0/0	0

* Remaining pigs at 49 days post infection.

**data for 4 weeks post infection was not available for serotypes 1, 5 and 7.

Serological findings

Table 2.3 summarizes the serological and PCR results obtained throughout the experiment. No seroconversion was observed in pigs infected with serotype 5 and 12 or in the non-infected control group at any time point using both ELISA tests. Pigs from these groups were also PCR negative at day 49 post infection. The Multi-APP ELISA detected seroconversion one week earlier than the serotype-specific Swinecheck® APP ELISA tests in pigs infected with serotypes 1, 3, and 10. Both ELISA tests detected seroconversion at 2 weeks post-infection in pigs infected with serotypes 7 and 15. At day 49 post-infection, the Multi-APP ELISA detected a higher number of positives in groups infected with serotypes 1, 3, 10, and 15 compared with the serotype-specific Swinecheck® APP ELISA. Both ELISA tests detected 8 out of 9 positive pigs infected

with serotype 7 at this time point. Seroconversion varied greatly depending on the serotype (Figure 2.1). Lower seroconversion rate was observed in groups inoculated with serotypes 1, 3 and 10, whereas 89 and 100% seroconversion was detected in groups inoculated with serotypes 7 and 15, respectively. The number of pigs with positive tonsil PCR at day 49 post-infection was consistently lower than the number of pigs with positive antibody titers detected by Multi-APP ELISA tests in all groups (Table 2.3). The rough data showing the performance of each assay obtained when testing samples throughout the experiment are seen in table 2.4.

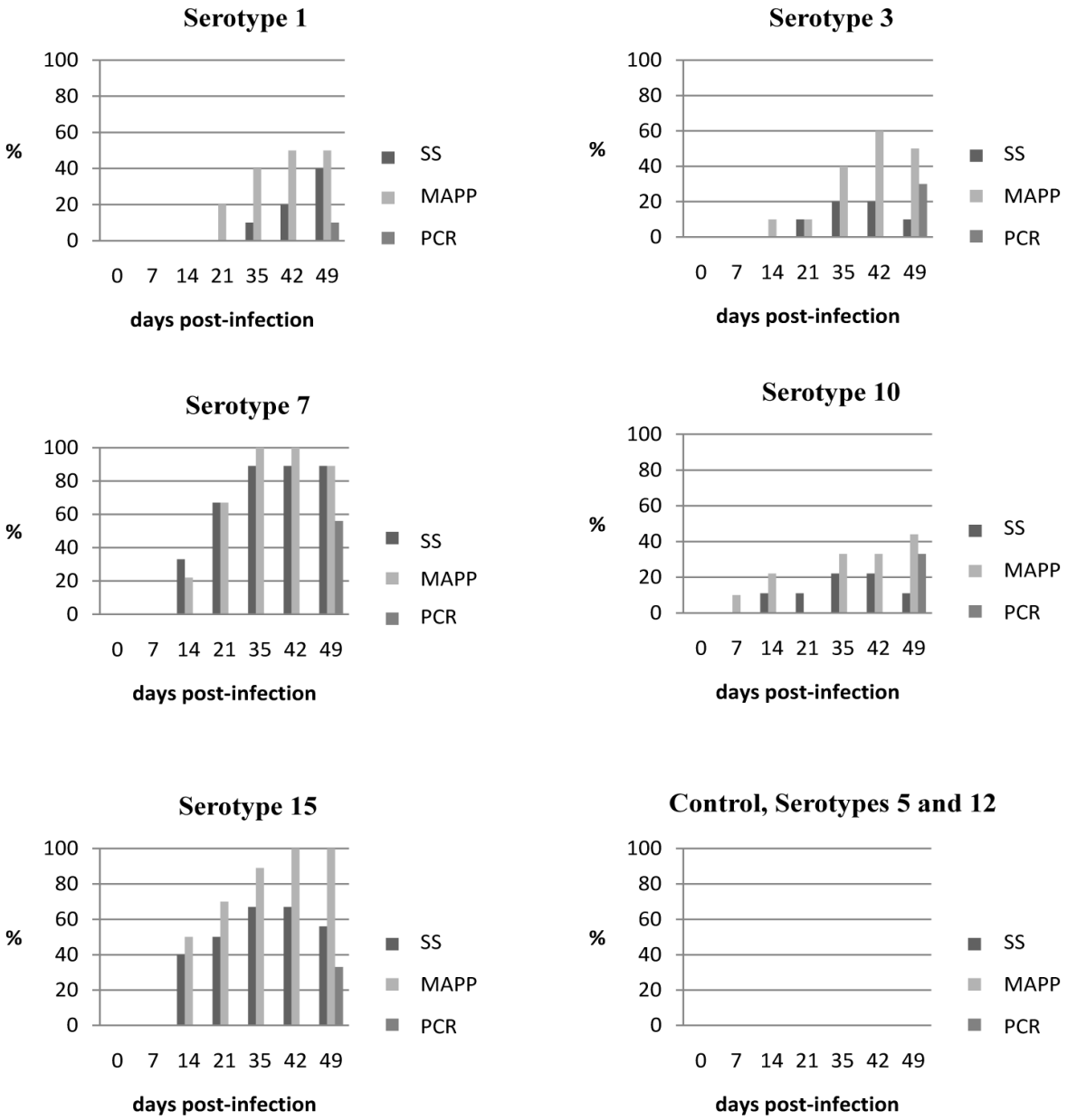

Figure 2.1 – Percentage of pigs detected by the Multi-APP (MAPP) and serotype-specific (SS) ELISA tests on different days post infection and by PCR on the day 49.

Table 2.4 – Individual ELISA and PCR results obtained for pigs involved in this study.

Strain	Pig #	PCR_Tonsil swab (dpi)								Sero-specific 1-9-11 ELISA (dpi)								Multi-APP ELISA (dpi)								PCR*
		0	7	14	21	28	35	42	49	0	7	14	21	28	35	42	49	0	7	14	21	28	35	42	49	(49dpi)
S	7	-	-	-	-	-	-	-	-	-	-	-	-	nd	-	-	-	-	-	-	-	nd	-	-	-	-
E	12	-	-	-	-	-	-	-	-	-	-	-	-	nd	-	-	-	-	-	-	-	nd	-	-	-	-
R	27	-	-	-	-	-	-	-	-	-	-	-	-	nd	-	-	-	-	-	-	-	nd	-	-	-	-
O	34	-	-	-	-	-	-	-	-	-	-	-	S	nd	+	+	+	-	-	-	-	nd	+	+	+	-
T	35	-	-	-	-	-	-	-	-	-	-	-	S	nd	S	+	+	-	-	-	+	nd	+	+	+	-
Y	42	-	-	-	-	-	-	-	-	-	-	-	-	nd	-	-	-	-	-	-	-	nd	-	-	-	-
P	43	-	-	-	-	-	-	-	-	-	-	S	-	nd	S	S	S	-	-	-	+	nd	+	+	+	-
E	48	-	-	-	-	-	-	-	-	-	-	-	-	nd	-	+	+	-	-	-	-	nd	-	+	+	-
	64	-	-	-	-	-	-	-	-	-	-	-	+	nd	+	+	+	-	-	-	-	nd	+	+	+	+
1	71	-	-	-	-	-	-	-	-	-	-	-	-	nd	-	-	-	-	-	-	-	nd	-	-	-	neg
Strain	**Pig #**	**PCR_Tonsil swab (dpi)**								**Sero-specific 3-6-8-15 ELISA (dpi)**								**Multi-APP ELISA (dpi)**								**PCR***
		0	7	14	21	28	35	42	49	0	7	14	21	28	35	42	49	0	7	14	21	28	35	42	49	**(49dpi)**
S	1	-	-	-	-	-	-	-	-	-	-	-	-	-	S	S	S	-	-	-	-	+	+	+	+	-
E	6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
R	8	-	-	-	+	-	-	-	-	nd	-	-	-	S	+	+	S	-	-	-	-	+	+	+	+	+
O	26	-	-	-	-	-	-	-	-	-	-	-	-	-	-	S	S	-	-	-	-	+	+	-	+	-
T	29	-	-	-	-	-	-	-	-	-	-	-	-	-	-	S	nd	-	-	-	-	-	-	-	-	+
Y	31	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P	50	-	-	-	-	-	-	-	-	-	-	-	-	S	S	S	S	-	-	-	-	+	+	+	+	-
E	52	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
	55	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	60	-	-	+	-	-	-	-	-	-	-	S	+	+	+	+	+	-	-	+	+	+	+	+	+	-
Strain	**Pig #**	**PCR_Tonsil swab (dpi)**								**Sero-specific 4-7 ELISA (dpi)**								**Multi-APP ELISA (dpi)**								**PCR***
		0	7	14	21	28	35	42	49	0	7	14	21	28	35	42	49	0	7	14	21	28	35	42	49	**(49dpi)**
S	15	-	+	-	+	nd	-	-	-	-	-	+	+	nd	+	+	+	-	-	+	+	nd	+	+	+	+
E	17	-	-	-	-	nd	-	-	-	-	-	+	+	nd	+	+	+	-	-	+	+	nd	+	+	+	-
R	18	-	-	-	-	nd	-	-	-	-	-	S	+	nd	+	+	+	-	-	-	+	nd	+	+	+	+
O	25	-	-	-	-	nd	-	-	-	-	-	-	+	nd	+	+	+	-	-	-	+	nd	+	+	+	-
T	37	-	+	-	+	nd	-	-	-	-	-	-	nd	nd	S	S	neg	-	-	-	nd	nd	+	+	+	+
Y	46	-	-	-	-	nd	-	-	-	-	-	-	+	nd	+	+	+	-	-	-	+	nd	+	+	+	-
P	54	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D
E	58	-	-	-	-	nd	-	-	-	-	-	-	-	nd	+	+	+	-	-	-	-	nd	+	+	+	-
	69	-	+	-	-	nd	-	-	-	-	-	+	-	nd	+	+	+	-	-	+	-	nd	+	+	+	+
7	76	-	-	-	+	nd	-	-	-	-	-	-	+	nd	+	+	+	-	-	-	+	nd	+	+	+	+

Table 2.4 (cont.) – Serological and PCR results obtained by the different tests evaluated throughout the experiment.

Strain	Pig #	PCR_Tonsil swab (dpi)								Sero-specific 10 ELISA (dpi)								Multi-APP ELISA (dpi)								PCR* (49dpi)
		0	7	14	21	28	35	42	49	0	7	14	21	28	35	42	49	0	7	14	21	28	35	42	49	
S	21	-	-	-	-	nd	-	+	-	-	-	-	-	-	S	-	S	-	-	-	-	-	+	-	+	-
E	22	-	-	-	-	nd	-	-	-	-	-	-	-	+	-	S	-	-	-	-	-	+	-	+	-	-
R	33	-	+	-	-	nd	-	-	-	-	-	-	S	S	S	S	S	-	-	-	-	-	-	+	+	+
O	36	-	-	-	-	nd	-	-	-	-	S	+	+	+	+	+	S	-	+	+	-	-	+	-	+	+
T	40	-	-	-	-	nd	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y	62	-	-	-	-	nd	-	-	-	-	-	-	nd	-	-	-	-	-	-	-	-	-	-	-	-	-
P	63	-	-	-	-	nd	-	-	-	-	-	S	S	-	+	+	+	-	-	+	-	nd	+	+	+	-
E	65	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D
	70	-	-	-	-	nd	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10	73	-	-	-	-	nd	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Strain	**Pig #**	**PCR_Tonsil swab (dpi)**								**Sero-specific 3-6-8-15 ELISA(dpi)**								**Multi-APP ELISA (dpi)**								**PCR* (49dpi)**
		0	7	14	21	28	35	42	49	0	7	14	21	28	35	42	49	0	7	14	21	28	35	42	49	
S	11	-	-	-	-	nd	-	-	-	-	-	-	+	+	+	+	+	-	-	-	+	+	+	+	+	+
E	38	-	-	-	-	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D
R	45	-	-	-	-	nd	-	-	-	-	-	-	S	+	+	+	+	-	-	+	+	+	+	+	+	+
O	53	-	-	+	-	nd	-	-	-	-	-	+	+	+	+	+	+	-	-	+	+	+	+	+	+	-
T	59	-	-	-	-	nd	-	-	-	-	-	+	nd	+	+	+	+	-	-	+	-	+	+	+	+	-
Y	61	-	-	-	-	nd	-	-	-	-	-	+	+	+	+	+	S	-	-	+	+	+	+	+	+	-
P	67	-	-	-	-	nd	-	-	-	-	-	+	+	+	+	+	+	-	-	+	+	+	+	+	+	-
E	68	-	-	-	-	nd	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	-
	77	-	-	-	-	nd	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+
15	78	-	-	-	-	nd	-	-	-	-	-	-	-	S	S	S	S	-	-	-	-	+	+	+	+	-
Strain[§]	**Pig #**	**PCR_Tonsil swab (dpi)**								**Sero-specific 1-9-11 BIOVET (dpi)**								**Multi-APP ELISA (dpi)**								**PCR* (49dpi)**
		0	7	14	21	28	35	42	49	0	7	14	21	28	35	42	49	0	7	14	21	28	35	42	49	
N	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
E	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
G.	9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C	20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
O	23	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
N	30	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
T	51	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
R.	66	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	80	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

D = Dead pig; Nd = not determined; S = suspect; * PCR results obtained when testing tonsils fragments obtained at necropsy moment 49 days post infection; § Serotype 5 and 12 remained negative when tested by all tests throughout the experiment.

Survival Analysis

The time from inoculation to seroconversion was considered the 'time to event' variable in the survival analysis. Animals that did not seroconvert by the end of the experiment were censored. The Multi-APP ELISA tended to detect seroconversion one week earlier than the serotype-specific Swinecheck® APP ELISA tests (Figure 2.2). The median survival time for seroconversion using the Multi-APP ELISA test (28 days) was significantly lower (p=0.04) than the median obtained using the Swinecheck® APP ELISA test (49 days). The median survival is the time (expressed in days) when half the inoculated pigs are expected to seroconvert. It means that the chance of seroconverting beyond that time is 50%.

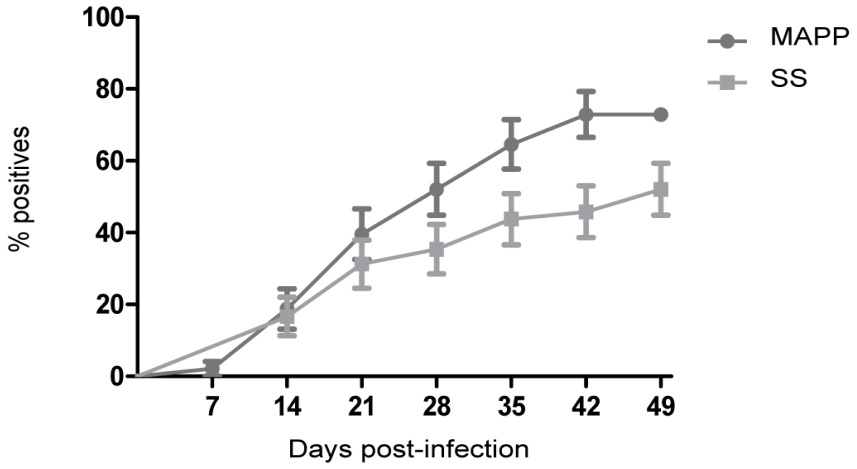

Figure 2.2 – Survival analysis based on the time between inoculation and detection of seroconversion using the Multi-APP and serotype-specific ELISA tests (suspects in the serotype-specific ELISA tests were considered negatives).

Discussion

Actinobacillus pleuropneumoniae serological monitoring is commonly used in the U.S. to prevent the introduction of subclinically infected pigs into naïve populations. The prevalence of *A. pleuropneumoniae* infected swine herds is now relatively low in the U.S. The serotype-specific Swinecheck® APP ELISA tests were for many years utilized as the gold standard for serological screening. Although these tests generate reliable and specific data regarding *A. pleuropneumoniae* infection, the costs associated with screening for several *A. pleuropneumoniae* serotypes are prohibitive for many swine herds. In 2005, two new *A. pleuropneumoniae* screening tests were introduced into the

U.S. – the Chekit APP-ApxIV® ELISA and the Multi-APP ELISA offered by the Veterinary Diagnostic Laboratory at the University of Montreal. The Chekit APP-ApxIV® ELISA targets antibodies against the Apx IV toxin produced by *A. pleuropneumoniae* during infection, whereas the Multi-APP ELISA and the serotype-specific Swinecheck® APP ELISA tests target antibodies against the LC-LPS. With many tests available for *A. pleuropneumoniae* serological screening, discordant results were a frequent finding among field veterinarians. The objective of this study was to compare the performance of all available serological tests to detect *A. pleuropneumoniae* subclinical infection under identical experimental conditions.

Subclinically infected pigs are the main source of *A. pleuropneumoniae* introduction into naïve populations. In this study we aimed to reproduce subclinical infection by exposing pigs to a sublethal dose of *A. pleuropneumoniae* (10^6 CFU/ml) (Fittipaldi et al., 2003a). Subclinical infection was accomplished in groups infected with serotypes 1, 3, 7, and 15. Pigs infected with serotype 5 and 12 did not become infected, whereas pigs infected with serotype 10 showed evident clinical signs 24-48 hours post infection. All pigs were infected with the same dose, indicating that virulence and colonization varies depending on the serotype used. The unsuccessful infection of pigs with serotypes 5 and 12 was unexpected, since these serotypes are commonly associated with disease in the field. Seroconversion has been successfully generated in pigs inoculated with 10^{10} CFU/ml of *A. pleuropneumoniae* serotype 12 (Grondahl-Hansen et al., 2003), whereas typical pleuropneumonia lesions have been reproduced following experimental infection with 10^8-10^9 CFU/ml of *A. pleuropneumoniae* serotype 5 (Ueda et

al., 1995). The dose required to reproduce a subclinical infection for serotypes 5 and 12 may be higher than that used in this study.

The development of severe clinical signs following infection with serotype 10 was also unexpected. A query of *A. pleuropneumoniae* isolation from clinical cases at the Minnesota Veterinary Diagnostic Laboratory from 2002 to 2008 revealed that serotype 10 was not isolated from clinical cases in the U.S. during this period. The few isolates that were classified as serotype 10 by IHA were not confirmed as *A. pleuropneumoniae* based on toxin profile, genotyping, and sequencing of the 16rRNA gene (Oliveira et al., 2007). Although serotype 10 is not commonly isolated from field cases in the U.S., we decided to include it in the study due to the fact that a few swine herds reported seroconversion to this serotype in the absence of clinical infection. The serotype 10 strain utilized to infect pigs in this study was obtained from Dr. Blackall's collection, and it may differ from strains that are currently circulating in the U.S. There is no information in the literature regarding the experimental infection of pigs with this serotype.

Our initial design included weekly testing of serum samples post-infection using the serotype-specific Swinecheck® APP ELISA tests, the Multi-APP ELISA tests, and the Chekit APP-ApxIV®-based offered by IDEXX; however, IDEXX interrupted commercialization of the Apx IV ELISA test right after we started the trial and we were only able to test the source herd and Day 0 samples with this kit. Consequently, we were only able to compare the performance of the serotype-specific Swinecheck® APP ELISA tests and the Multi-APP ELISA for days 0 to 49. Although both of these tests detect

antibodies to the LC-LPS of APP, there were important differences in the consistency of results obtained weekly for each infected group (Figure 2.1). More importantly, there were important differences in sensitivity to detect early seroconversion.

The Multi-APP ELISA tended to detect seroconversion one week earlier than the serotype-specific Swinecheck® APP ELISA tests (Figure 2). It also tended to be more consistent in maintaining positive and negative results for individual pigs in consecutive weeksm (Table 2.4). The serotype-specific Swinecheck® APP ELISA tests first identified true positives (as defined by Multi-APP ELISA) as suspects, followed by weak positive results in the subsequent week. Inconsistencies in suspects, positives, and negative results were mainly observed with the serotype-specific Swinecheck® APP ELISA tests detecting antibodies against the serogroup 1-9-11 and serotype 10. The Multi-APP ELISA contains a mix of LC-LPS antigens obtained from all *A. pleuropneumoniae* serotypes and may represent a more complete antigen pool for detection of early seroconversion compared with the serotype-specific ELISA tests, which contains a single antigen for each serotype.

Our results demonstrated that the serotype-specific Swinecheck® APP ELISA tests formulated to detect antibodies against the serogroup 3-6-8 can also detect antibodies against the serotype 15. S/P ratios were in fact higher for pigs infected with serotype 15 compared with those infected with serotype 3 (data not shown). This data raised the question whether other cross-reactions could occur using the serotype-specific Swinecheck® APP ELISA tests. To address this questions, we cross-tested all serum

samples obtained at day 49 post-infection from pigs infected with serotypes 1, 3, 7, 10, and 15 with the serotype-specific ELISA tests 1-9-11, 2, 3-6-8, 4-7, 10, and 12. No cross-reactions were observed, confirming that these tests specifically detect the serogroups indicated on each kit.

Although tonsil swabs were collected weekly for isolation and PCR, both tests failed to confirm infection throughout the experiment. Tonsil swabs utilized for isolation also carried the normal flora, which made identification of *A. pleuropneumoniae* colonies a difficult task. Although *A. pleuropneumoniae* has been successfully isolated from the tonsils of infected pigs (Ueda et al., 1995), this was not reproduced in this study. Techniques such as immunomagnetic isolation are known to improve the chances of isolating *A. pleuropneumoniae* from tonsils (Gagné et al., 1998). However, our laboratory was not set up to run this technique. *A. pleuropneumoniae* was successfully isolated from the lungs of pigs that developed pleuropneumonia, corroborating the value of isolation to confirm *A. pleuropneumoniae* infection in the presence of typical lesions. PCR failed mostly due to the use of incorrect swabs for sample collection. The gel media in the bottom of the swab interfered with the PCR reaction and severely affected the sensitivity of the technique. PCR of tonsil fragments collected during necropsy at 49 days post15 infection did yield positive results. These results, in addition to the serological results, were useful to confirm infection of pigs inoculated with *A. pleuropneumoniae* serotypes 1, 3, 7, 10, and 15 and to confirm that pigs inoculated with serotypes 5 and 12 did not become infected.

The number of pigs per group that were positive by tonsil PCR was lower than the number of pigs that were seropositive at 49 days post infection. There was no direct correlation between a positive PCR results and a positive serological result. These differences may be associated with the fact that pigs with positive serological results may have cleared *A. pleuropneumoniae* from the tonsils, whereas pigs with negative serological results but positive tonsil PCR results could be recently infected through nose-to-nose contact within a group. Although we failed to detect *A. pleuropneumoniae* in tonsil swabs by PCR, we did detected APP DNA in the saliva obtained from ropes hanged in the rooms with pigs infected with serotypes 7 and 10 at 1 week post infection (data not shown). These results suggest that shedding was likely more active during the acute phase of infection, and that tonsil PCR would still have been a considerable alternative diagnostic method to troubleshoot unexpected serological results during this phase, provided proper swabs are used for sample collection.

Based on the results obtained in this study, we recommend that the Multi-APP ELISA test be used for serological screening of pigs prior to introduction into naïve populations. The serotype-specific Swinecheck® APP ELISA tests can be used to identify the serotype circulating in the herd following the detection of positives by the Multi-APP test. Positive serum samples based on the Multi-APP ELISA results may test negative with the serotype-specific Swinecheck® APP ELISA. Re-test of these samples 1-2 weeks later with the serotype-specific ELISA compensates for the difference in sensitivity among these tests.

CHAPTER 3

Development of a real-time polymerase chain reaction assay for detection of *Actinobacillus pleuropneumoniae.*

OVERVIEW

The detection of pigs subclinically infected with *Actinobacillus pleuropneumoniae* (APP) has become an important step in eliminating this agent from infected swine herds and a critical measure to avoid the introduction of positive pigs into naïve populations. Therefore, a highly sensitive and specific test to accurately detect subclinically infected pigs is needed. In this study, a real-time PCR assay was developed to detect the *apx*IV toxin gene of *A. pleuropneumoniae* in clinical samples. The real-time PCR was validated based on analytical sensitivity and specificity and testing of tonsil samples collected from pigs subclinically infected by experimental exposure to 7 clinically relevant *A. pleuropneumoniae* serotypes. Results obtained by the real-time PCR were compared with those obtained using a previously described gel-based PCR assay also targeting the *apx*IV gene. The newly developed real-time PCR detected a single *A. pleuropneumoniae* genome copy and 17 subclinically infected pigs. The gel-based PCR detected 10 genome copies and 15 subclinically infected pigs. Both tests specifically detected *A. pleuropneumoniae* and were negative when tested with DNA from unrelated bacterial pathogens frequently isolated from swine tissues. Non-specific amplifications observed when DNA extracted from tonsil tissues were tested using the gel-based PCR were not an issue with the real-time PCR. The newly developed *A. pleuropneumoniae* real-time PCR is a sensitive and specific method for identification of subclinically infected pigs and can be used as a tool to evaluate the efficacy of control and eradication strategies.

Introduction

A wide range of diagnostic tools can be used to identify swine pleuropneumonia, an important disease caused by *Actinobacillus pleuropneumoniae* (APP). (Bossé et al., 2002; Chiers et al., 2001; de la Puente-Redondo et al., 2000; Gram et al., 1998; Grondahl-Hansen et al., 2003; Hernanz et al., 1999; Inzana et al., 2001; Klausen et al., 2007; Lebrun et al., 1999; Lo et al., 1998; Lobin et al., 1982; Nielsen, 1982; Rodriguez et al., 1990; Savoye et al., 2000; Schaller et al., 2001; Sidibé et al., 1993a; Dreyfus et al., 2004).This pathogen is highly contagious (Bossé et al., 1993; Taylor 1999) and responsible for large economic losses in the swine industry worldwide (Fenwick et al., 1994). Detection of *A. pleuropneumoniae* in clinical samples with classical pleuropneumonia lesions is very straightforward and easily accomplished by bacterial isolation (Jacobsen et al., 1995; Taylor, 1999). Isolation from tonsil swabs, on the other hand, is difficult and frequently unsuccessful due to overgrowth by the resident tonsillar flora (Chiers et al., 2001; Sidibé et al., 1993; Taylor 1999). Identification of *A. pleuropneumoniae* carriers or subclinically infected pigs is of great importance to troubleshoot unexpected serological results, accurately evaluate the success of control and eradication strategies, and prevent the introduction of carriers into naïve populations.

Considering the limitations of bacterial isolation for the detection of carriers, PCR has become the technique of choice for the detection of *A. pleuropneumoniae* in tonsil samples. Several gel-based PCR techniques have been developed for the detection of *A. pleuropneumoniae*, including a ready to use kit commercialized by Adiavet (Adiàgene,

St. Brieuc, France). These tests target different genes, including *oml*A (Savoye, 2000), *dsb*E-like (Chiers et al., 2001), *aro*A (Hernanz et al., 1999), *cps-cpx* (Lo et al., 1998) and *apx*IVA (Schaller et al., 2001) and they vary in sensitivity and specificity (Fittipaldi et al., 2003). None of the test currently available for *A. pleuropneumoniae* diagnostics utilizes real-time technology. Real-time PCR is known to be less labor intensive, usually more specific, and very likely more sensitive due to the computerized detection of light emission instead of human eye detection of a gel band (Mackay, 2004; Smith et al., 2009).

Most U.S. swine herds are now negative for *A. pleuropneumoniae* and a highly sensitive and specific diagnostic test for identification of subclinically infected pigs is needed. PCR testing on tonsil tissues and swabs is frequently used by field veterinarians to detect carriers (Oliveira et al., 2007). At the MVDL, a modified method of a previously described gel-based PCR has been offered routinely for the detection of *A. pleuropneumoniae* in tonsil samples (Schaller et al., 2001). In our experience, non-specific amplification is occasionally observed when testing DNA extracted directly from tonsils and from cultures obtained from tonsil swabs. In order to overcome these limitations, we have developed and validated a real-time PCR to improve the detection of *A. pleuropneumoniae* in tonsil samples obtained from subclinically infected pigs.

Material and Methods

Bacterial strains

A total of 41 bacterial strains were used to validate the newly developed real-time PCR,including 15 reference strains of *A. pleuropneumoniae*, 14 reference strains of *Haemophilus parasuis*, and 13 other unrelated pathogens frequently isolated from swine tissues (Table 3.1). *A. pleuropneumoniae* reference strains for serotypes 1–6 were obtained from the American Type Culture Collection (ATCC). Reference strains for serotypes 7–15 were kindly provided by Dr. Blackall at the Department of Primary Industries and Fisheries, Animal Research Institute, Australia. (Blackall et al., 2002). The *Haemophilus parasuis* reference strains were kindly provided by Dr. Rapp-Gabrielson (Kielstein et al., 1992). All the other bacterial species were obtained from the Minnesota Veterinary Diagnostic Laboratory (MVDL) collection.

Table 3.1 - Bacterial strains used for validation of the real-time PCR assay for detection of *Actinobacillus pleuropneumoniae*.

STRAIN	SEROTYPE	ID	Real-time PCR RESULT
*Actinobacillus pleuropneumoniae**	1	27088	+
*Actinobacillus pleuropneumoniae**	2	27089	+
*Actinobacillus pleuropneumoniae**	3	27090	+
*Actinobacillus pleuropneumoniae**	4	33378	+
*Actinobacillus pleuropneumoniae**	5	33377	+
*Actinobacillus pleuropneumoniae**	6	35590	+
Actinobacillus pleuropneumoniae†	7	WF83	+
Actinobacillus pleuropneumoniae†	8	405	+
Actinobacillus pleuropneumoniae†	9	CVJ-13261	+
Actinobacillus pleuropneumoniae†	10	22009	+
Actinobacillus pleuropneumoniae†	11	56153	+
Actinobacillus pleuropneumoniae†	12	1096	+
Actinobacillus pleuropneumoniae†	13	N273	+
Actinobacillus pleuropneumoniae†	14	3906	+
Actinobacillus pleuropneumoniae†	15	HS143	+
*Mycoplasma hyopneumoniae**	-	25095	-
*Mycoplasma hyorhinis**	-	25591	-
*Mycoplasma hyosynoviae**	-	17981	-
Haemophilus parasuis‡	1	4	-
Haemophilus parasuis‡	2	SW 140	-
Haemophilus parasuis‡	3	SW 114	-
Haemophilus parasuis‡	4	SW 124	-
Haemophilus parasuis‡	5	Nagasaki	-
Haemophilus parasuis‡	6	131	-
Haemophilus parasuis‡	7	174	-
Haemophilus parasuis‡	9	D74	-
Haemophilus parasuis‡	10	H555	-
Haemophilus parasuis‡	11	H465	-
Haemophilus parasuis‡	12	H425	-
Haemophilus parasuis‡	13	84-17975	-
Haemophilus parasuis‡	14	84-22113	-
Haemophilus parasuis‡	15	84-15995	-
Streptococcus suis§	-	D06-002347	-
Actinobacillus suis§	-	D06-002228	-
Actinobacillus indolicus§	-	D05-064134	-
Actinobacillus minor§	-	D06-002338	-
Actinobacillus porcinus§	-	D05-062291	-
Pasteurella multocida§	-	D09-002228	-
Bordetella bronchiseptica§	-	D06-002352	-
Escherichia coli§	-	D06-002351	-
Salmonella choleraesuis§	-	D05-022917	-
Clostridium perfringens (?)	-	Ark ISO-3	-

* ATCC
† Blackall et al (2002)
‡ Kielstein and Rapp-Gabrielson (1992)
§ Minnesota Veterinary Diagnostic Laboratory

Clinical samples

Clinical samples tested in this study were obtained from pigs experimentally infected with *A. pleuropneumoniae*. This experiment was approved by the University of Minnesota Institutional Animal Care and Use Committee (IACUC) (protocol#0806A37381). Eighty pigs were divided into 8 groups of 10 pigs each. Pigs from groups 1 to 7 were inoculated intranasally (1ml/nostril) with a sub-lethal dose of $1x10^6$ CFU/ml of the following *A. pleuropneumoniae* serotypes: 1, 3, 5, 7, 10, 12 and 15 (Fittipaldi 2003). Pigs in group 8 were not inoculated and remained as negative controls. All pigs were obtained from a source free of *A. pleuropneumoniae* based on clinical history, tonsil PCR (Schaller et al., 2001) and serology (Dreyfus et al., 2004). DNA extracted from tonsil samples (0.3 g) collected at 49 days post-infection (necropsy) were tested by the gel-based (Schaller et al., 2001) and by the newly developed real-time PCR assay. Duplicate swabs were also cultured in 5% sheep blood agar at 37°C during 24h using a *Staphylococcus sp*. streak for *A. pleuropneumoniae* isolation (Jacobsen et al., 1995, Taylor 1999). In addition to the samples obtained by pigs experimentally infected with *A. pleuropneumoniae,* a total of 86 clinical samples submitted to the Veterinary Diagnostic Laboratory of the University of Minnesota was also tested by both tests.

DNA extraction

DNA was extracted from all bacterial cultures listed in Table 3.1 and from tonsil fragments collected from pigs inoculated with *A. pleuropneumoniae* serotypes 1, 3, 5, 7,

10, 12 and 15 (10 pigs per serotype) at 49 days post-infection, and 10 non-inoculated negative control pigs. Pure cultures were placed into a microcentrifuge tube containing 300µL of PBS (phosphate buffer saline) and vortexed for 1minute. After vortexing, each tube was centrifuged at 13,000 rpm for 3 min to obtain a pellet. Following centrifugation, the supernatant was discarded and cell pellets were resuspended in 200µL of DNA extraction matrix (PrepMan Ultra Applied Biosystems, Foster City, CA.) and vortexed for 15 seconds. Samples were then boiled for 20 minutes and centrifuged again at 13,000 for 3 minutes. Fifty microliters of the supernatant of each tube was transferred to a new tube containing 50µL of RNA-free water. These solutions were kept at 4°C overnight before being tested and used as templates for the gel-based and real-time reactions (Oliveira et al., 2007). DNA extraction from tonsil tissues was performed using a commercial kit (DNeasy Blood and Tissue kit, Qiagen, Valencia, CA) following the manufacture's protocol.

Primers and probes used in the* real-time PCR *assay

The 300 bp non-specific band generated when testing DNA extracted from tonsil obtained from known negative pigs (negative control group) was sequenced and aligned with the *apx*IV gene of *A. pleuropneumoniae* using a software (Tamura et al., 2007). By using this approach, areas of similarity between the *apx*IV and the non-specific sequence were avoided during primer and probe designs. Primers and probes specifically targeting the *apx*IV gene of *A. pleuropneumoniae* were designed using a software Primer3 (http://frodo.wi.mit.edu/primer3/) following the standard parameters set by the program.

The expected size of the amplicon generated by the designed primers was 218 bp. The real-time PCR probe (Taqman® Universal PCR Master Mix, Applied Biosystems, Foster City, CA) was labeled with the reporter dye FAM and the quencher TAMRA. Sequences for the primers and probes developed in this study are shown in Table 3.2.

Table 3.2 – Oligonucleotide primers and probe used in the real-time PCR assay.

TARGET GENE AND SIZE	**PRIMER OR PROBE**	**SEQUENCE (5'-3')**
*apx*IV (218bp)	Forward	GCG GAG AGG GTA ATG ATA CG
	Reverse	CTC GGC TAA ACC AAA GTT CG
	Probe	AGG ACA CGG ACA GGA TAT CG

***Real-time PCR* conditions**

The real-time assay was standardized using DNA extracted from a pure culture of *A. pleuropneumoniae* strain ATCC 27088. Real-time reactions were performed on a thermocycler *(*ABI 7500 Fast Cycler, Applied Biosystems, Foster City, CA) equipped with sequence detection system software using a standardized 25µL reaction mixture. The reaction mixture included 12.5µL of PCR mix containing 5x RT-PCR Buffer (QuantiFast™ Probe PCR + ROX vial kit, Qiagen) and final concentration of 2.5 mM MgCl, 0.5µL ROX dye, 0.25µM of each primer and probe, 5.5µL of sterile RNase-free water, and 5µL of template DNA. Incubation conditions consisted of an initial denaturation at 95ºC for 3 min, followed by 40 cycles of 95ºC for 15 sec and 50 ºC for 1 min. Fluorescent signals were detected in FAM channel. The line for calculating the threshold cycle number (CT) for this channel was assigned to a fixed value intersecting

the amplification curves in the linear region of the logarithmic plot. Any samples showing a fluorescent signal above this line were regarded as positive.

Gel-based PCR conditions

The original protocol for the detection of the *apx*IV gene using a gel-based test is a nested PCR (Schaller et al., 2001). In order to reduce the risks of contamination usually associated with nested PCR reactions (Dubosson et al., 2004) we have opted to utilize only the first reaction described at the original protocol for routine testing at the MVDL. The gel-based PCR was conducted in a thermocycler (GeneAmp PCR System 9700 Applied Biosystem, Foster City, CA) in a final volume of 25µL. The PCR mix contained 12 µL of PCR mix (HotStart Taq polymerase - containing buffer and dNTPs - Qiagen kit) containing 250 units HotStarTaq DNA Polymerase, 10 x PCR Buffer, 5 x Q-Solution, 25 mM MgCl2), a final concentration of 0.25µL of each primer, 9.4µL sterile RNase-free water and 2µL of template DNA. PCR conditions consisted of an initial denaturation at 95ºC for 15 min, followed by 35 cycles of 94ºC for 30 sec, 52 ºC for 30 sec and 72 ºC for 30 sec, with a final hold of 72 ºC for 10 minutes. Sequences for the primers used in the gel-based PCR are shown in Table 3.3. Amplicons obtained using this protocol were sequenced to confirm the amplification of the correct target.

Table 3.3 – Oligonucleotide primers used in the gel-based PCR assay.

TARGET GENE AND SIZE	PRIMER OR PROBE	SEQUENCE (5'-3')
*apxI*V (422bp)	APX 4 A 1L APX 4 A 1R	TGG CAC TGA CGG TGA TGA GGC CAT CGA CTC AAC CAT

Analytical sensitivity and specificity

Analytical sensitivity for the gel-based and real-time PCR tests was defined by testing 10-fold dilutions of the pure culture DNA extracted from *A. pleuropneumoniae* strain ATCC 27088. Number of copies detected was defined by DNA quantification (1 genome copy = 5 fg) (Sanderson et al., 1992). Analytical specificity for both tests was evaluated by testing DNA from 15 reference strains for *A. pleuropneumoniae* serotypes and 27 unrelated bacterial pathogens strains of 13 species frequently isolated from swine tissues, including *Haemophilus parasuis, Streptococcus suis, Actinobacillus suis, Actinobacillus indolicus, Actinobacillus minor, Actinobacillus porcinus, Pasteurella multocida, Bordetella bronchiseptica, Escherichia coli, Clostridium perfringens, Mycoplasma hyopneumoniae, Mycoplasma hyorhinis, Mycoplasma hyosynoviae,* and *Salmonella choleraesuis*.

Statistical analysis

The results obtained by the two tests (gel-based and real-time PCRs) were compared by the Kappa coefficient agreement calculation (κ). Discordant results were evaluated by the Chi-square binomial test.

Results

Real-time PCR assay

Primers and probes designed for this study are listed on Table 2. Relevant characteristics and optimal PCR conditions for the gel-based PCR and real-time PCR assays are given in Table 3.4. The real-time protocol provided results in one hour, whereas 2 hours and 40 min were needed to obtain results with the one-step gel-based PCR protocol.

Table 3.4 – Running conditions characteristics for the gel-based PCR and real-time PCR assays

CHARACTERISTICS	GEL-BASED PCR	REAL-TIME PCR
Thermocycler	9700 Thermocycler	7500 Fast Cycler
Reaction vessel	0.2 µL PCR reaction tubes	96-well reaction plate
Reaction volume	25 µL	25 µL
Use of internal probes	no	Yes
Sample volume	2 µL	5 µL
PCR Master Mix	12 µL Hot Start Taq 0.25 µM primer forward 0.25 µM primer reverse 9.4 µL sterile RNAse-free water - -	12.5 µl QuantiFast Probe PCR Mix 0.25 µM of primer forward 0.25 µM of primer reverse 1.25 µl sterile RNase-free water 0.25 µM of probe[1] 1 µl Fast ROX dye
PCR program	Hold: 95˚C/15 min 35 cycles: 94˚C/30 sec 94˚C/30 sec 52˚C/30 sec 72˚C/30 sec 2nd hold: 72˚C/10 min	Hold: 95ºC/3 min 40 cycles: 95ºC/15 sec 50ºC/1 min
Duration of amplification	90 min	60 min
Detection	Gel electrophoresis (30 min)	Fluorescence monitoring (real-time)

Analytical sensitivity and specificity

The real-time test detected one single copy of *A. pleuropneumoniae* genome (5fg) per reaction, whereas the gel-based test detected a minimum of 10 genome copies (50fg) per reaction. Both tests specifically detected *A. pleuropneumoniae* when using template DNA from unrelated bacterial species listed on Table 3.1.

Clinical samples

A summary of the results obtained from samples of pigs experimentally infected with *A. pleuropneumoniae* using the gel-based and the newly developed real-time PCR real-time PCR is shown in Table 3.5. The agreement between the gel-based and the real-time PCR tests was 93% with kappa value (κ) of 0.775. Thirteen pigs were positive for *A. pleuropneumoniae* based on both methods. The real-time PCR detected 4 pigs in groups infected with *A. pleuropneumoniae* serotypes 3, 7, 10 and 15 that were not detected by the gel-based PCR. Two pigs infected with serotype 7 and 10 were positive by gel-based PCR and negative by real-time. Fifty-seven pigs, including all pigs from groups infected with serotypes 5, 12, and the non-inoculated control groups were negative by both methods. There was no statistically significant difference between both tests when tests results were compared using the Chi-square test (α=0.05). When testing clinical samples submitted to the MVDL, there was a 100% of agreement between both tests. The gel-based PCR generated a non-specific band of 300 bp when run with DNA extracted from tonsils. This band was sequenced and found to be 78% identical to *Psychrobcter sp.* when

compared to sequences available at the GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi). *A. pleuropneumoniae* was not isolated from any of the tonsil samples tested.

Table 3.5 – Summary of the results obtained from samples of pigs experimentally infected with *A. pleuropneumoniae* when tested by gel-based and real-time PCR.

RESULTS BY		**GEL-BASED PCR**		
		Pos	Neg	Total
REAL-TIME	Pos	13	4	17
	Neg	2	57	59
	Total	15	61	76*

*Four pigs died during the experiment

Table 3.6 – Summary of the results obtained from clinical samples submitted to the MVDL when tested by gel-based and real-time PCR.

RESULTS BY		**GEL-BASED PCR**		
		Pos	Neg	Total
REAL-TIME	Pos	2	0	2
	Neg	0	85	85
	Total	2	85	87

Discussion

The aim of this study was to develop a highly sensitive and specific real-time PCR test for the detection of *A. pleuropneumoniae* in tonsils of subclinically infected pigs. We have opted for the real-time format for several reasons. By designing 2 new primers and 1 probe we were able to target 3 species-specific regions at the *apx*IV gene and eliminate the non-specific amplifications observed with the gel-based test (Schaller et al., 1999; Schaller et al., 2001). By using a TaqMan® probe, we were able to increase sensitivity and reduce the workload involved with running a gel to obtain results. Besides being slightly more sensitive and highly specific, the new real-time PCR has allowed us to provide high-throughput testing with faster results.

In this study we have elected to test tonsil samples, as these are the most likely to be submitted for testing at the MVDL. In our experience, *A. pleuropneumoniae* is easily isolated from lungs with characteristic lesions of pleuropneumonia. In these cases, PCR has little value to confirm infection. As most swine herds in the US are free of *A. pleuropneumoniae*, tonsil sample testing has become a very important step in defining herd status. PCR is widely used by field veterinarians to troubleshoot unexpected serological results and to evaluate the outcome of control strategies.

Bacterial isolation is usually regarded as the gold-standard for *A. pleuropneumoniae* diagnostics (Marsteller et al., 1999). In this study, we were unable to isolate *A. pleuropneumoniae* from tonsil fragments collected from subclinically infected pigs due to overgrowth by the commensal flora. Considering the limitations of isolating

APP from tonsil samples, we compared the results obtained using the new real-time PCR with those obtained using a modified version (only first reaction of a nested PCR) of a previously published gel-based PCR (Schaller et al., 2001). We have been using this gel-based PCR for the past 5 years at the MVDL with good results. However, we occasionally observed the presence of a non-specific band at 300bp, especially when testing tonsil swabs or biopsies. This same band was observed when we tested DNA extracted from tonsil fragments collected from experimentally infected pigs and more interestingly from the negative control group. By sequencing the non-specific band obtained from one of the known negative pigs (obtained from a source negative for *A. pleuropneumoniae* based on PCR and serology and included in the non-inoculated control group), we were able to align this sequence with the *apx*IV gene sequence and design primers and probes that avoided similar regions in both sequences. The non-specific sequence was identified as *Psychrobacter sp.* This bacterium is commonly isolated from swine manure and proteinaceous food. Contact with fecal matter was probably the cause for the amplification of the non-specific band by the gel-base PCR.

There was substantial agreement (κ=0.775) between the results obtained using the gel-based PCR and the real-time PCR. The new real time PCR was slightly more sensitive than the gel-based PCR tests, detecting one single genome copy compared to 10 genome copies detected by the latter. Although the real-time test detected a higher number of subclinically infected pigs at 49 days post-infection compared to the gel based test, this difference was not statistically significant. The differences in sensitivity observed could be potentially explained by the quantity of template DNA used per

reaction in each test and differences in the detection method (fluorescence versus UV light detection) (Heid et al., 1996; Mackay, 2004). The number of clinical samples tested in this study were limited by submissions to the MVDL. Only 2 infected pigs were detected using the real-time and the gel-based PCR. Further validation using samples collected from endemically infected and APP-free populations will likely reinforce the advantages of this test for detection of carrier pigs. Both the real-time and the gel-based test specifically detected *A. pleuropneumoniae*. No non-specific amplifications were detected when using the real-time PCR. All pigs in the non-inoculated control group were negative when tested using the real-time PCR, including those that had non-specific bands generated by the gel-based test. Negative PCR results using both tests for the non-inoculated control groups were expected, as these pigs remained serologically negative throughout the duration of experimental infection study. Pigs inoculated with a sublethal dose of *A. pleuropneumoniae* serotypes 5 and 12 were also negative by both PCR tests and did not seroconvert, which indicates that infection with these serotypes have failed. Successful infections with serotypes 5 and 12 have been reported (Andresen et al., 2002; Bossé et al., 2002; Gottschalk et al., 1994; Klausen et al., 2002; Stenbaek et al., 1997); however, higher doses were used for inoculation.

This study reports the development of a rapid, sensitive, and specific real-time PCR assay using new primers and TaqMan® technology targeting the detection and amplification the *apx*IV gene of *A. pleuropneumoniae*. When dealing with mostly negative populations, the detection of subclinically infected carriers becomes very important to avoid the introduction of these animals into naïve herds. Although the

difference in sensitivity between the newly developed real-time and the gel-based test was minimal, we feel that it is an important step towards better assessment of herd status. Besides being slightly more sensitive, the real time test has several other advantages that are key for routine laboratory testing: it is less labor intensive, provides faster results, and is less subjective regarding the interpretation of results. This new test will greatly add to the investigation of unexpected serological results and evaluation of control and eradication strategies.

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