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Influenza A virus antibody in porcine oral fluid and its diagnostic applications

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Influenza A virus antibody in porcine oral fluid and its diagnostic applications

by

Yaowalak Panyasing

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

DOCTOR OF PHILOSOPHY

Major: Veterinary Microbiology (Preventive Medicine)

Program of Study Committee:
Jeffrey J. Zimmerman, Major Professor
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Iowa State University
Ames, Iowa

2013

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DEDICATION

To my parents
Paisan and Muey Panyasing

To my husband
Dr. Apisit Kittawornrat

To my major professor
Professor Dr. Jeffrey J. Zimmerman
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ABSTRACT

Influenza A virus (IAV) is capable of infecting a wide variety of avian and mammalian species, including swine. The control of IAV in swine populations is complicated by the fact that the virus is endemic in contemporary herds and may circulate in any age group. The detection of IAV has historically been based on testing individual pig nasal swab (virus detection) or serum (antibody detection) specimens. While individual pig sampling is adequate for the diagnosis of clinical IAV infections, the collection of adequate numbers of individual pig samples is too costly and labor-intensive for routine influenza surveillance or large-scale ecological studies. Therefore, the general question addressed in this dissertation is whether oral fluid specimens could be used to surveil IAV infections as an alternative to individual animal sampling. More specifically, the aim of this research was to evaluate an IAV oral fluid antibody enzyme-linked immunosorbent assay (ELISA) for the detection of IAV nucleoprotein (NP) antibody and its use in surveillance of swine populations. This question was addressed in the logical series of experiments described below.

The initial objective was to determine whether diagnostic levels of IAV NP antibodies could be detected in swine oral fluid specimens by adapting the serum ELISA protocol to the oral fluid matrix (Chapter 3). The NP antibody ELISA was selected because the NP is highly conserved among IAV subtypes. The procedure for performing the NP blocking ELISA on oral fluid was modified from the serum testing protocol by changing sample dilution, sample volume, incubation time, and incubation temperature. The detection of NP antibody was evaluated using pen-based oral fluid samples \( n = 182 \) from pigs inoculated with either influenza A virus subtype H1N1 or H3N2 under experimental conditions and followed for 42 days post inoculation (DPI). NP antibodies in oral fluid were detected from DPI 7 to 42 in all inoculated groups, i.e., the mean sample-to-negative (S/N) ratio of influenza-inoculated pigs was significantly different \( p < 0.0001 \) from uninoculated controls (unvaccinated or vaccinated-uninoculated groups) through this period. Oral fluid vs. serum S/N ratios from the same pen showed a correlation of 0.796 (Pearson correlation coefficient, \( p < 0.0001 \)). The results showed that oral fluid samples from influenza virus-infected pigs contained detectable levels of NP antibodies for \( \geq 42 \) DPI.
The availability of serum and oral fluid NP ELISAs provided the tools necessary to describe the kinetics of IAV NP antibody (IgM, IgA, and IgG) in serum and oral fluid specimens from animals of defined IAV infection status (Chapter 4). A significant oral fluid IgM response was only detected in unvaccinated groups. The maximum oral fluid IgM response in these groups was detected at DPI 8, after which it rapidly declined. Oral fluid IgA was detected in both vaccinated and unvaccinated groups on DPI 6. Levels of oral fluid IgA remained relatively stable through DPI 42. Oral fluid IgG responses in both vaccinated and unvaccinated groups were detected by DPI 8 and remained stable through DPI 42. IgM responses in serum and oral fluid were highly correlated in unvaccinated groups (r = 0.810), as were serum and oral fluid IgG responses in both unvaccinated (r = 0.839) and vaccinated (r = 0.856) groups. In contrast, the correlation between serum and oral fluid IgA was weak (r ~ 0.3), regardless of vaccination status. The results from this study demonstrated that NP-specific IgM, IgA, and IgG antibody were detectable in serum and oral fluid and their ontogeny was influenced by vaccination status, the time course of the infection, and specimen type.

The feasibility of IAV surveillance in the field was evaluated using pre-weaning oral fluid samples from litters of piglets in four ~12,500 sow, IAV-vaccinated, breeding herds (Chapter 5). All four herds were considered endemically infected with IAV based on historic diagnostic data. Oral fluid samples were collected from 600 litters prior to weaning and serum samples from their dams after weaning. Litter oral fluid samples were tested for IAV by virus isolation, quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR), RT-PCR subtyping, and sequencing. Commercial NP ELISA kits and NP isotype-specific assays (IgM, IgA, and IgG) were used to characterize NP antibody in litter oral fluid and sow serum. All litter oral fluid specimens (n = 600) were negative by virus isolation. Twenty-five oral fluid samples were positive by qRT-PCR, based on screening (Laboratory 1) and confirmatory testing (Laboratory 2). No hemagglutinin (HA) and neuraminidase (NA) gene sequences were obtained, but matrix (M) gene sequences were obtained for all qRT-PCR-positive samples submitted for sequencing (n = 18). Genetic analysis revealed that all M genes sequences were identical (GenBank accession no. KF487544) and belonged to the triple reassortant influenza A virus M gene (TRIG M) previously identified in swine. The
proportion of IgM- and IgA-positive samples was significantly higher in sow serum and litter oral fluid samples, respectively ($p < 0.01$). Consistent with the extensive use of IAV vaccine, no difference was detected in the proportion of IgG- and blocking ELISA-positive sow serum and litter oral fluids. This study supported the use of oral fluid sampling as a means to conduct IAV surveillance in pig populations and demonstrated the inapparent circulation of IAV in piglets.
CHAPTER 1. DISSERTATION ORGANIZATION

This dissertation consists of 6 chapters. The first chapter describes the organization of the dissertation. Chapter 2 is a review of “The production and distribution of antibody in the pig oro-respiratory system” to be submitted for publication in Veterinary Immunology and Immunopathology. Chapter 3, “Detection of influenza A virus nucleoprotein antibodies in oral fluid specimens from pigs infected under experimental conditions using blocking ELISA” was published in Transboundary and Emerging Diseases. Chapter 4, “Kinetics of influenza A virus nucleoprotein antibody (IgM, IgA, and IgG) in serum and oral fluid specimens from pigs infected under experimental conditions” has been published in Vaccine. Chapter 5, ”Influenza A virus (IAV) surveillance using pre-weaning piglet oral fluid samples” has been submitted for publication in Preventive Veterinary Medicine. The references, tables, and figures associated with each chapter follow the discussion section. The final chapter contains the General Conclusions of the dissertation.
CHAPTER 2. REVIEW OF THE PRODUCTION AND DISTRIBUTION OF ANTIBODY IN THE PIG ORO-RESPIRATORY SYSTEM

Yaowalak Panyasing, James A. Roth, Jeffrey J. Zimmerman

To be submitted to Veterinary Immunology and Immunopathology

ABSTRACT

Under constant exposure to microorganisms, the oro-respiratory immune system contains and controls microbes using a combination of non-specific (innate) and specific (acquired) immune mechanisms. Herein we focus on the processes by which antibody is produced and distributed to mucosal tissues and secretions in the pig oro-respiratory system. The humoral immune response of the porcine oro-respiratory system is similar to other mammalian species in terms of the process of producing and distributing antibody to mucosal surfaces. That is, antibodies are produced by plasma cells located in salivary glands and underlying mucosal epithelium or arrive by passive diffusion from the capillaries in the oro-respiratory tract. However, the porcine immune system also has unique features, e.g., an inverted lymph node structure and the predominance of \( \gamma\delta \) T lymphocytes in blood and mucosal tissues. A basic comprehension of this process is fundamental to achieving an understanding disease pathogenesis, vaccine development, and disease diagnostics. However, it should be recognized that our understanding of some aspects of this process are limited, e.g., mechanisms related to the production of antibody by lymphoid tissues in salivary glands and questions on the specific receptors or mechanisms that assist antibody transport into pig lungs.
INTRODUCTION

Terrestrial animals are routinely and continuously exposed to a wide-ranging variety of nonpathogenic and pathogenic microorganisms via oral and airborne exposures. For an animal to survive, much less thrive, under this constant barrage of microorganisms, the oro-respiratory immune system must be able to control their replication and spread within the body. This is done using a combination of non-specific (innate) and specific (acquired) mechanisms. The innate immunity of the oral cavity and respiratory tract is based on physical barriers (mucosa, cilia, and mucins), chemical barriers (antimicrobial enzymes e.g., lysozymes; antimicrobial peptides, e.g., defensins and cathelicidins), phagocytic cells (neutrophils, macrophages) and the complement system (Murphy, 2012). In contrast, acquired immunity is a two-pronged system capable of producing cell-mediated and humoral immune responses. Cell-mediated immunity generally involves cytotoxic T cells and eliminates intracellular microorganisms via apoptosis. Humoral immunity is mediated by antibody focused on the destruction of extracellular pathogens and antigens by neutralization, opsonization, and complement activation (Murphy, 2012). Unlike innate immunity, acquired immunity gains momentum with exposure and time, and develops a memory capable of generating a faster and more effective response to subsequent antigenic exposure.

Antibodies on mucosal surfaces and in mucosal secretions of the oro-respiratory tract are an important line of acquired defense. Thus, anti-viral antibody on mucosal surfaces is the pig's primary immune defense against foot-and-mouth disease virus, pseudorabies virus, and influenza A virus (Francis and Black, 1983; Kimman et al., 1992; Larsen et al., 2000). In mammals, five classes (isotypes) of immunoglobulin (IgM, IgA, IgG, IgE, and IgD) are recognized. These differ in the heavy chain portion of the molecule (µ, α, γ, ε, and δ, respectively). Pigs produce IgM, IgA, IgE and six subclasses of IgG (IgG1 through 6), but do not produce IgD (Butler et al., 2009). Knowledge of how the humoral oro-respiratory immune systems functions to protect pigs is basic to an understanding of herd immunity, vaccine efficacy, and diagnostic medicine.
ANATOMY AND HISTOLOGY

In this section we provide a brief overview of the porcine anatomy (Figure 1) and histology relevant to the production and distribution of antibody in the pig oro-respiratory system (Figure 2).

Oral cavity
The areas of the oral cavity exposed to frictional and shearing forces (gingiva, dorsal surface of the tongue, and hard palate) are lined by parakeratinized to completely keratinized stratified squamous epithelium, with an underlying layer of dense irregular collagenous connective tissue (Gartner and Hiatt, 2007). The remainder of the oral cavity is covered by nonkeratinized stratified squamous epithelium overlying a looser type of dense, irregular, collagenous connective tissue.

Pigs have three major salivary glands: parotid, mandibular, and sublingual. Minor salivary glands exist as scattered lobules in the mucosa and submucosa of the buccal, labial, palatal, and lingual regions (Nickel et al., 1979; Stembirek et al., 2012). An adult pig produces approximately 15 liters of saliva daily (König and Liebich, 2004; Stembirek et al., 2012). Histologically, salivary glands consist of secretory and duct portions. A secretory unit or "acinus" (plural: acini) is composed of secretory cells (serous and mucous cells) surrounded by myoepithelial cells. The secretory cells making up the acini produce serous, mucous, or mixed secretions. The parotid salivary gland produces serous secretions, the mandibular and sublingual glands produce mixed secretions (serous and mucous), and the minor salivary glands produce mucous secretions. Acinar secretions are modified as they move through the intercalated, striated, and excretory ducts to the oral cavity. In addition, striated duct cells synthesize the secretory component or "polymeric immunoglobulin receptor" that binds the J-chain of dimeric IgA and IgM (Tandler et al., 2001). Swine and human salivary glands share common anatomical characteristics and physiological functions (Zhou et al., 2010), but not all aspects have been researched in pigs. For example, it is not known whether the duct-associated lymphoid tissue (DALT) in the minor salivary glands of humans is also present in pigs (Liebler-Tenorio and Pabst, 2006). Salivary glands differ in the composition of their
secretions and in the quantity of antibody produced. For example, the density (number per
mm$^3$) of IgG, IgA, IgM, and IgD secreting-cells was significantly higher in human parotid
than submandibular salivary glands, but IgA-secreting cells were predominant in both
(Korsrud and Brandtzaeg, 1982).

**Respiratory system**

Pig lungs are divided into seven lobes. Each lung (left and right) is divided into cranial
(apical), middle (cardiac), and caudal (diaphragmatic) lobes, but the right lung has an
intermediate (accessory) lobe in addition to the other three (Frandson and Spurgeon, 1992).
Distinct from humans, the pig has a “tracheal bronchus” that connects the right cranial lobe
of the lung directly to the trachea (Maina and van Gils, 2001).

The respiratory system can be broadly divided into three parts: conducting airways (nasal
cavity, paranasal sinus, pharynx, larynx, trachea, and bronchi), transitional airways
(bronchioles), and the structures associated with gas exchange (alveoli). Within the
conducting airways, the vestibular region of the nasal cavity is lined by stratified squamous
epithelium, whereas the remainder of the nasal cavity, the pharynx, larynx, trachea, and
bronchi, are covered by pseudostratified ciliated columnar epithelium containing variable
numbers of goblet cells and serous cells (Gagné and Martineau-Doizé, 1993; VanAlstine,
2012). The nasal and tracheal subepithelial connective tissue (lamia propria) is highly
vascularized and contains seromucous glands and lymphoid elements, including occasional
lymphoid nodules, mast cells, and plasma cells (Gartner and Hiatt, 2007). Within the
transitional airways, the ciliated cells that predominate in the anterior bronchioles are
gradually replaced by non-ciliated bronchiolar epithelium in the posterior bronchioles
(Tizard, 2009). Normal bronchioles lack goblet cells, but possess non-ciliated, dome-shaped
epithelial columnar cells with short, blunt microvilli known as "Clara cells" (Tizard, 2009).
At the level of gas exchange, the alveoli are lined with a single layer of pneumocytes (type I
and the larger type II).

**Lymph nodes**

Three major lymph centers are located in the head and neck: parotid, mandibular, and retropharyngeal. The dorsal half of the head is drained by the parotid lymph
center, the oral cavity and salivary glands by the mandibular lymph center, and the pharynx, larynx, and cranial part of the trachea by the retropharyngeal lymph center (König and Liebich, 2004). In the lung, lymph is drained to the bronchial and tracheobronchial lymph nodes located at the tracheal bifurcation (König and Liebich, 2004; Riquet et al., 1999).

Anatomically, lymph nodes are divided into cortex, paracortex, and medulla. Histologically, lymph nodes consist of a reticular network filled with lymphocytes, macrophages, and dendritic cells (Tizard, 2009). The structure of the porcine lymph node is inverted compared to most mammals, with the medulla at the periphery and the cortex in the center (Binns and Pabst, 1994; Tizard, 2009). Lymph enters the cortex via afferent lymphatic vessels, passes from the center of the node to the periphery, and exits via efferent lymphatic vessels. Lymphocytes in the blood stream enter the lymph node via specialized blood vessels termed "high endothelial venules" and return to the bloodstream through the high endothelial venules of the paracortex area rather than the efferent lymphatic vessels (Binns and Pabst, 1994). For this reason, very few lymphocytes are found in pig lymph.

Primary lymphoid follicles, composed primarily of resting B cells, are located in the cortex. The primary lymphoid follicle becomes a secondary lymphoid follicle when the B cells proliferate and differentiate in response to antigenic stimulation. Histologically, a secondary lymphoid follicle consists of a mantle zone containing resting B cells (Chianini et al., 2001) and a germinal center composed of (1) a light zone where immunoglobulin class switching and memory B cell formation occur and (2) a dark zone where B cells proliferate and undergo the process of somatic hypermutation (MacLennan, 1994). It follows that the lymph nodes of germ-free piglets lack germinal centers until they experience an antigenic challenge (Anderson et al., 1974).

**Tonsils** Tonsils are mucosa-associated lymphoid tissue involved in initiating an immune response to pathogens entering the oral cavity and respiratory system. Pigs have five tonsils: the pharyngeal tonsil on the roof of the nasopharynx, tubal tonsils at the pharyngeal openings of the auditory tubes, paraepiglottic tonsils located craniolaterally to the base of the epiglottis, tonsils of the soft palate, and lingual tonsils at the root of the tongue.
Tonsils do not have afferent lymph ducts, but lymph is drained by the lymphatic network to the mandibular lymph center (Belz, 1998; Binns and Pabst, 1994; Trautmann and Fiebiger, 1957).

Tonsils are characterized as follicular or non-follicular by the presence or absence of blind pouches (tonsillar crypts) that function in immune surveillance. Tonsillar crypts extend from the surface to the underlying lymphoid tissue (Belz, 1998; Trautmann and Fiebiger, 1957). The tonsils of the soft palate, tubal tonsils and paraepiglottic tonsils are follicular, whereas the pharyngeal and lingual tonsils are non-follicular (Trautmann and Fiebiger, 1957). All tonsils are covered by non-keratinized stratified squamous epithelium, but in follicular tonsils this layer extends through the first portion of the crypt after which it transitions to thinner stratified squamous epithelium highly infiltrated by lymphocytes (lymphoepithelium). Within the crypts, occasional membranous cells (M cells) and goblet cells are found between epithelial cells and lymphocytes, macrophages, and plasma cells migrate within and through the lymphoepithelium (Horter et al., 2003). Lymphoid follicles containing B cells surrounded by T cells are located beneath the lymphoepithelium, (Bianchi et al., 1992; Salles et al., 2000).

**Bronchus-associated lymphoid tissue (BALT)** BALT most commonly consists of a single lymphoid follicle in the lamina propria of the bronchi. The follicle bulges into the lumen of the bronchus, giving it a dome-like or nodular structure (Huang et al., 1990; Pabst and Tschernig, 2010). The BALT is covered by specialized non-ciliated epithelium with cytoplasmic projections on its surfaces and infiltrations of lymphocytes within and beneath the epithelium (Huang et al., 1990). In some species, e.g., rabbits, M cells specialized for antigen uptake are also present (Bienenstock and Johnston, 1976). In pigs, the flattened epithelial cells covering the BALT are hypothesized to be M cell precursors (Huang et al., 1990). In pigs, antigenic exposure stimulates BALT development. Thus, BALT is absent in germ-free pigs and found only in a proportion (30 to 50%) of healthy pigs (Pabst and Gehrke, 1990). Pigs infected with *Actinobacillus pleuropneumoniae* (Delventhal et al., 1992a, b) or *Mycoplasma hyopneumoniae* (Sarradell et al., 2003) had significantly more BALT structures compared to uninfected pigs. Similar to tonsils, BALT has no afferent lymphatic ducts and
lymph is drained to the bronchial and tracheobronchial lymph nodes (Binenstock and McDermott, 2005; Pabst and Binns, 1995; Pabst and Tschering, 2002).

CELLS ASSOCIATED WITH ANTIGEN PROCESSING AND PRESENTATION

**Dendritic cells**

Dendritic cells are a heterogeneous population of antigen-presenting cells that prime naïve T cells and/or B cells (Tizard, 2009). Dendritic cells can be found in tonsil, spleen, lymph nodes, and blood (Jamin et al., 2006). Although there is limited information on their distribution in the porcine respiratory tract, dendritic cells are abundant both below the basement membrane and within the tracheal epithelium (Bimczok et al., 2006). Dendritic cells on the epithelial mucosa serve as immunological sentinels, i.e., they capture, internalize, process, and present antigen to naïve T cells. Intraepithelial dendritic cells commonly have long dendrites extending toward the tracheal lumen that probably relate to this immunological surveillance function (Bimczok et al., 2006). Dendritic cells play a role in T cell responses and homing characteristics (Saurer et al., 2007). For example, interferon (IFN)-γ and interleukin (IL)-12 produced by dendritic cells stimulate T helper1 development (cell-mediated immunity), whereas IL-4, IL-10 and/or IL-13 favor a T helper 2 response (antibody formation) (Murphy, 2012; Raymond and Wilkie, 2004). The effect of porcine dendritic cells on the differentiation of T regulatory cells has not been described (Summerfield and McCullough, 2009).

**Macrophages**

Although found throughout the body, the lung is populated by two specific types of macrophages: pulmonary alveolar macrophages (PAM) and pulmonary intravascular macrophages (PIM) (Winkler and Cheville, 1987). Macrophages are antigen-presenting cells, but they are less efficient at this function than dendritic cells (Tizard, 2009). Once loaded with antigen, alveolar macrophages cross the alveoli and pass to regional lymph nodes where they interact with T and B cells to initiate an immune response (Constant et al., 2002). In young pigs, PIMs number ~14,000 per mm³ of lung parenchyma and cover ~16% of the total lung capillary surface (Winkler and Cheville, 1987; Wrinkler, 1988). Analogous to Kupffer cells in their function, PIMs serve to clear particles within the circulatory system and are a major component of the host's systemic defense mechanism (Staub, 1994). PIMs
have less phagocytic activity than PAMs and little is known of their activity as antigen-presenting cell (Chitko-McKown et al., 1991).

CELLS ASSOCIATED WITH ANTIBODY PRODUCTION

**T cells**  
T cells are found in the peripheral circulation and lymphoid tissues throughout the body (Belz, 1998; Chianini et al., 2001) and facilitate antibody production by their interactions with B cells. Porcine T cells are divided into two broad types based on the presence of αβ or γδ cell membrane receptors. αβ T cells only recognize antigens presented by the major histocompatibility complex (MHC) molecule, whereas γδ T cell antigen recognition is not limited to MHC presentation (Charerntantanakul and Roth, 2007; Yang et al., 1996). αβ T cells can be further divided into CD4+ and CD8+ subtypes. CD4+ T cells are involved in antibody production and predominate during bacterial and parasitic infections. CD8+ T cells are involved in cell-mediated immune responses and primarily function in antiviral responses (Charerntantanakul and Roth, 2007).

**B cells**  
B cells, the progenitor of antibody-secreting plasma cells, are primarily found in the follicular areas of the lymphoid organs and infrequently in blood (Bianchi et al., 1992). Each mature B cell possesses external receptors that recognize and bind a specific antigen. Once bound, antigen is internalized, processed, and returned to the cell surface as a peptide:MHC complex molecule. B cells are then activated by T helper cells that recognize the same epitope. Activated B cells proliferate, and differentiate into antibody-secreting plasma cells and long-lived memory cells (Murphy, 2012).

ANTIBODY PRODUCTION

The adaptive immune response begins when antigens are recognized by dendritic cells, macrophages, or other antigen-presenting cells. In the oral cavity and respiratory tract, these cells are located on the mucosal surfaces and in the organized lymphoid tissues (tonsils and BALT). Although this response initiates both cell-mediated and humoral immune responses, our focus is on the latter.
The priming of naïve T cells in tonsil, BALT and lymph nodes by antigen-presenting cells in the presence of specific cytokines leads T cells to undergo clonal expansion and differentiation (Raymond and Wilkie, 2004). In the presence of IL-4, IL-10, and/or IL-13, naïve T cells become T helper 2 cells, a CD4+ T cell subtype that promotes B cell proliferation and differentiation into antibody-secreting plasma cells (Murphy, 2012; Raymond and Wilkie, 2004). The humoral immune response is initiated when a T helper 2 cell recognizes the peptide:MHC complex molecule on the surface of a B cell ("linked recognition"). This event triggers the T helper 2 cell to synthesize B cell stimulatory molecules, including the T cell-bound CD40 ligand and secretory effector molecules IL-4, IL-5, and IL-6. The interaction between the T cell CD40 ligand and the B cell CD40 receptor, in the presence of IL-4, IL-5 and IL-6, stimulates B cell proliferation and differentiation into plasma cells or memory B cells (Murphy, 2012).

Alternatively, B cells in the lamina propria of mucosal tissue can become IgA-secreting plasma cells without the participation of T helper cells by direct stimulation with B cell activating factor (BAFF), proliferation-inducing ligand (APRIL), and/or IL-17 (Doreau et al., 2009; Lawson et al., 2011; Puga et al., 2010). BAFF and APRIL are secreted by dendritic cells or epithelial cells, whereas IL-17 is a pro-inflammatory cytokine produced mainly by T helper 17 cells (Harrington et al., 2005; Lawson et al., 2011). Porcine T helper 17 cells differentiate from CD4+ T cells in the presence of transforming growth factor-β (TGF-β) and IL-6/or IL-1β (Kiros et al, 2011; Stepanova et al., 2012).

Plasma cells and memory B cells in tonsils can locomote to lacrimal glands, nasal mucosa, salivary glands, and bronchial submucosal glands (Brandtzaeg, 2007; Inoue, 1999) or migrate to the urogenital tract via the circulatory system (Bergquist et al., 1997; Czerkinsky et al., 1994). Plasma cells and memory B cells in BALT can locomote to the bronchial submucosal glands and bronchial lumen (Bienenstock and Clancy, 2005; Brandtzaeg, 2007). Primed B cells can also migrate via draining lymphatic ducts to regional lymph nodes or, if they enter the blood stream, to the lung interstitium or remote mucosal tissues, e.g., gastrointestinal tract (Bienenstock and Clancy, 2005; Brandtzaeg, 2007; Saif, 1996). The destination of these cells is determined by a homing mechanism based on complementary adhesion molecules on
the lymphocyte (integrin) and the high endothelial venules (addressin) in tandem with matched lymphocyte chemokine and tissue-specific chemokine receptors (Bourges et al., 2007; Kunkel and Butcher, 2003). For example, B cells and T cells that strongly express integrin $\alpha_4\beta_1$ and chemokine subfamily CC receptor 10 (CCR10) on their surface preferentially "home" to tissues expressing addressin vascular cell adhesion molecule-1 (VCAM-1) and chemokine subfamily CC ligand 28 (CCL28) (Bourges et al., 2004, 2007; Kunkel and Butcher, 2003; Xu et al., 2003). In pigs, CCL28 is abundant in the pharyngeal tonsils and trachea, while VCAM-1 is found in the pharyngeal tonsils, lamina propria of the respiratory tract, and blood vessels in the nasal mucosa (Bourges et al., 2004, 2007; Meurens et al., 2006).

**DISTRIBUTION AND TRANSPORT OF ANTIBODY INTO ORO-RESPIRATORY SYSTEM**

Oro-respiratory secretions and tissues contain both systemic and locally-produced IgM, IgA, and IgG (Holmgren, 1973; Korsrud and Brandtzaeg, 1982; Martinez-Tello et al., 1968). IgA predominates in upper respiratory tract secretions whereas IgG predominates in the lower respiratory tract (Morgan et al., 1980). Depending on antibody isotype and anatomical location, antibody reaches mucosal secretions via passive diffusion or receptor-mediated transport. Thus human parotid saliva contains >95% locally synthesized IgA, but whole saliva contains substantial amounts of IgG as a result of passive diffusion (Butler et al., 1990). The poly IgA (pIgR) and neonatal Fc (FcRn) receptors transport IgA and IgG, respectively, across epithelial surfaces in a variety of species (Ravetch, 1997).

**Antibody in the oral cavity**

Systemic antibody, primarily IgG but also monomeric IgA, passively diffuses from the circulatory system into the oral cavity via one of three routes: (1) from capillaries that lie beneath the epithelium of the oral mucosa; (2) via the gingival crevice, i.e., the space between the gingiva and the teeth, or (3) through the tight junctions between glandular cells of the salivary glands (Butler, 1967; Korsrud and Brandtzaeg, 1982; Naumova et al., 2013). Within the oral cavity, this antibody-rich transudate mixes with secretions from the major
and minor salivary glands (Challacombe and Shirlaw, 2005). Local production of antibody (IgM, IgA, IgG) occurs in plasma cells located in salivary glands, tonsils, and perhaps in duct-associated lymphoid tissue (Nair and Schroeder, 1986). Thus, either oral or Thiry-Vella loop inoculation of pigs with *Escherichia coli* strain 1261 or transmissible gastroenteritis virus resulted in the appearance of pathogen-specific IgM, IgA, and IgG-secreting plasma cells in mandibular and sublingual salivary glands (DeBuysscher and Berman, 1980; DeBuysscher and Dubois, 1978). Mucosal IgA-or IgM-secreting plasma cells typically express a small "J chain" polypeptide (15 kDa) that promotes formation of polymeric IgA and pentameric IgM (Brandtzaeg, 1974; Korsrud and Brandtzaeg, 1979). The transmembrane secretory (~80 kDa) component expressed on the basolateral surfaces of secretory epithelial cells acts as an epithelial receptor by binding the J chain of polymeric IgA or pentameric IgM and selectively transporting them across mucosal epithelium and into mucosal secretions (Brandtzaeg, 1974; Brandtzaeg and Prydz, 1984; Kacskovic, 2004). No binding of IgA or IgM was observed with epithelial cells lacking the secretory component (Brandtzaeg and Prydz, 1984). The anatomical distribution of the secretory component has not been described in pig salivary glands, but in human parotid and submandibular salivary glands, the secretory component is present in most acinar, intercalated, and striated duct cells, whereas the lining cells of excretory ducts lacks the secretory component (Korsrud and Brandtzaeg, 1982).

In human palatine tonsils, detection of immunoglobulin associated with the tonsil epithelium suggested active immunoglobulin transport, although the presence of the secretory component in the tonsillar epithelium has not been reported (Brandtzaeg et al., 1978; Tang et al., 1995). Whether or not active transport exists in tonsil, immunoglobulin, IgG in particular, is able to cross the tonsillar epithelium via passive diffusion and enter the tonsillar crypts (Brandtzaeg et al., 1978).

Duct-associated lymphoid tissue (DALT) surrounding the ducts of minor salivary glands is thought to play a role in local production of secretory antibody. This process occurs as a result of direct access to antigens via the short ducts of the minor salivary glands and remote antigenic stimulation as part of the common mucosal immune system (Nair and Schroeder,
Thus, minor salivary glands in humans produce approximately 30 to 35% of the total secretory IgA in oral secretions (whole saliva) (Crawford et al., 1975). However, no structural or physiological evidence of DALT associated with minor salivary glands has been described in pigs (Liebler-Tenorio and Pabst, 2006).

**Antibody in the respiratory system**

Transudates containing systemic IgG and monomeric IgA passively diffuse from the capillaries, pass between respiratory epithelial cells, and reach the apical surface of the respiratory mucosal surfaces (Morgan et al., 1980; Ramphal et al., 1979). Systemic IgG constitutes 78% of the total IgG in nasal secretions, 60% in trachea secretions, and 37% in bronchoalveolar secretions; the remainder consists of locally-produced IgG (Morgan et al.1980). In contrast, systemic IgA constitutes 1 to 2.5% of the IgA in respiratory secretions with the remainder composed of locally-produced IgA.

Local production of antibody occurs throughout the respiratory system, but the majority of plasma cells throughout the pig respiratory tract (with the exception of the lung parenchyma) produce IgA. IgG-producing plasma cells are more numerous than IgM-secreting cells in the lung and nasal mucosa, but the reverse is true in the trachea and bronchus (Bradley et al., 1976). The relative concentration of IgA vs. IgG in tracheal and bronchoalveolar secretions corresponds to the proportion of IgA- or IgG- producing plasma cells in the tracheal mucosa and lung, respectively (Bradley et al., 1976; Morgan et al., 1980). Thus, tracheal cultures synthesized more IgA than IgG, whereas the reverse was the case with lung tissue culture (Morgan et al., 1980). In pigs, age may also affect the content of respiratory secretions. Thus, the ratio of IgA to IgG was significantly greater in the nasal, tracheal, and bronchoalveolar secretions from sows than 18- to 20-week-old pigs (Morgan et al., 1980). IgM-producing plasma cells are also present in the respiratory tract, but little is known regarding their contribution to respiratory secretions (Bradley et al., 1976).

Two receptors are involved in the active transport of immunoglobulin into mucosal secretions of the respiratory tract. Specifically, the secretory component facilitates transport of IgA and the neonatal Fc receptor (FcRn) transports IgG. As previously discussed, the
secretory component is present in salivary glands, but it is also expressed in the nasal epithelium, mixed mucoserous glands beneath the nasal mucosa, and in the tracheobronchial lamina propria (Bourges et al., 2007). The location of the secretory component corresponds to the distribution of IgA in the respiratory tract (Bradley et al., 1976).

Neonatal Fc receptor (FcRn) is a 40 kDa major histocompatibility complex (MHC) class I-related molecule that binds to the Fc-domain of IgG in a pH-dependent manner (Ghetie and Ward, 2000; Stirling et al., 2005). FcRn is expressed in the epithelial cells of the ileum, jejunum, and duodenum of both neonatal and adult pigs (Stirling et al., 2005). In the first 24 to 36 hours after birth, neonatal piglets are able to absorbed antibodies in the dam's colostrum, but after gut closure FcRn receptors facilitate the transport of maternal IgG from the intestinal lumen into the piglet's circulatory system (Curtis and Bourne, 1971; Wangstrom et al., 2000). In the neonatal gut, IgG binding occurs at acidic pH and IgG release at neutral pH. Thus, maternal IgG is bound by FcRn receptors on the apical surface of the epithelial cells at the lower pH found in the intestinal lumen. Receptor-bound IgG then enters the cell by receptor-mediated endocytosis and is transported across the epithelial cell to the basolateral surface. Exposure to physiological pH promotes the release of IgG into the circulation. Once in the circulatory system, maternal antibody can cross into piglet's respiratory tract and its secretions (Bradley et al., 1976; Nechvatalova et al., 2005, 2011; Stirling et al., 2005). Thus, antigen-specific IgG and IgA were detected in bronchoalveolar secretions from piglets that ingested colostrum from sows vaccinated with an experimental antigen (Nechvatalova et al., 2011).

In the pig respiratory tract, the specific mechanism by which IgG crosses the alveolar epithelial barrier is uncertain (Spiekermann et al., 2002). However, FcRn has been detected in a variety of tissues, e.g., lung (bovine, human, mouse), liver (rodent, pig), kidney (pig), spleen (pig) and mammary gland (sheep, pig), and is postulated to modulate IgG transport at these sites (Blumberg et al., 1995; Ghetie and Ward, 2000; Kacskovics, 2004; Mayer et al., 2002; Mayer et al., 2004; Schnulle and Hurley, 2003; Spiekermann et al., 2002; Stirling et al., 2005). Although the specific mechanism by which IgG crosses the alveolar epithelial barrier has not been identified, FcRn-mediated IgG transport in the lower airway and alveoli
has been demonstrated in several species including rat, bovine, and cynomologus monkey (Bitonti et al., 2004; Kim and Malik, 2003; Mayer et al., 2004; Spiekermann et al., 2002).

CONCLUSIONS

The oro-respiratory tract is the site of entry for a tremendous number and variety of microorganisms. These must be controlled by the host immune system, if health is to be maintained. In particular, this discussion has focused on the means by which the humoral immune system responds to this exposure and populates the oro-respiratory tract with antibodies both from plasma cells in local tissues (salivary glands or mucosal epithelium) and from the circulatory system by passive diffusion. A better understanding of the mechanics and dynamics of this process has the potential to provide insight into improved pig health and welfare through better vaccines and improved diagnostics.

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Figure 1. Major salivary glands, tonsils, and lymph nodes of the pig oral cavity and respiratory system. 1. pharyngeal tonsil 2. tubal tonsil 3. paraepiglottic tonsil 4. tonsil of the soft palate 5. lingual tonsil 6. parotid salivary gland 7. mandibular salivary gland 8. sublingual salivary gland 9. parotid lymph node 10. mandibular lymph node 11. retropharyngeal lymph node 12. tracheobronchial lymph node (adapted with permission from Dyce et al., 2010)
Figure 2. Schematic of the processes resulting in the presence of antibodies on the mucosa and in mucosal secretions of the oro-respiratory system
CHAPTER 3. DETECTION OF INFLUENZA A VIRUS NUCLEOPROTEIN ANTIBODIES IN ORAL FLUID SPECIMENS FROM PIGS INFECTED UNDER EXPERIMENTAL CONDITIONS USING A BLOCKING ELISA

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SUMMARY

In commercial swine populations, influenza A virus is an important component of the porcine respiratory disease complex (PRDC) and a pathogen with major economic impact. Previously, a commercial blocking ELISA (FlockChek™ Avian Influenza Virus MultiScreen® Antibody Test Kit, IDEXX Laboratories, Inc., Westbrook, ME) designed to detect influenza A nucleoprotein (NP) antibodies in avian serum was shown to accurately detect NP antibodies in swine serum. The purpose of present study was to determine whether this assay could detect NP antibodies in swine oral fluid samples. Initially, the procedure for performing the NP blocking ELISA on oral fluid was modified from the serum testing protocol by changing sample dilution, sample volume, incubation time, and incubation temperature. The detection of NP antibody was then evaluated using pen-based oral fluid samples (n = 182) from pigs inoculated with either influenza A virus subtype H1N1 or H3N2 under experimental conditions and followed for 42 days post inoculation (DPI). NP antibodies in oral fluid were detected from DPI 7 to 42 in all inoculated groups, i.e., the mean sample-to-negative (S/N) ratio of influenza-inoculated pigs was significantly different (p < 0.0001) from un inoculated controls (unvaccinated or vaccinated-uninoculated groups) through this period. Oral fluid vs. serum S/N ratios from the same pen showed a correlation of 0.796 (Pearson correlation coefficient, p < 0.0001). The results showed that oral fluid samples from influenza virus-infected pigs contained detectable levels of NP antibodies for ≥ 42 DPI. Future research will be required to determine whether this approach could be used to monitor the circulation of influenza virus in commercial pig populations.
INTRODUCTION

In swine, influenza A virus (IAV) infection is traditionally described as an acute, upper respiratory disease with high morbidity, low mortality, and respiratory signs characterized by coughing, nasal and/or ocular discharge, sneezing, dyspnea, hyperthermia, anorexia, lethargy and weight loss (Brown, 2000; Janke, 1998; Loeffen et al., 1999; Richt et al., 2006). Influenza virus infection in individual swine may be diagnosed on the basis of clinical signs and postmortem lesions (Van Reeth et al., 2012). Biological samples (nasal swabs, bronchoalveolar lavage, serum, etc.) may be assayed by virus isolation, polymerase chain reaction (PCR), antibody-based assays, and other diagnostic techniques to support the diagnosis (Van Reeth et al., 2012).

While suitable for diagnostic evaluations of clinical outbreaks, individual animal testing is not amenable to IAV surveillance in large commercial swine populations. Alternatively, IAV infections have been detected using pen-based oral fluid samples collected under either experimental or field conditions and tested by reverse transcription (RT)-PCR (Detmer et al., 2011; Romagosa et al., 2011; Ramirez et al., 2012). Indeed, oral fluid-based diagnostics have been used extensively in human diagnostic medicine to detect and surveil a variety of infections, e.g., human immunodeficiency virus, mumps virus, measles virus, rubella virus, hepatitis C virus, and others (De Cock et al., 2004; De Cock et al., 2005; Holguín et al., 2009; Perry et al., 1993). Thus, oral fluid sampling could provide a more convenient method for the surveillance of influenza virus infections in confined animal feeding operations.

While PCR testing results reflect recent infection events, antibodies have the potential to inform regarding the history of infections in individuals and populations. Cameron and Carman (2005) state that most serum ELISAs can be modified to detect antibodies in oral fluid samples by changing the procedure, e.g., increasing the sample volume, increasing the incubation time, changing conjugate concentration, and changing cut-off values. A commercial ELISA designed to detect anti-influenza A nucleoprotein (NP) antibodies in avian serum was earlier shown to effectively detect NP antibodies in swine serum (Ciacci-Zanella et al., 2010). The purpose of present study was to determine whether diagnostic
levels of NP antibodies could also be detected in swine oral fluid specimens by adapting the serum ELISA protocol to the oral fluid matrix.

MATERIALS AND METHODS

Experimental design
The detection of NP antibodies over time in pen-based oral fluid samples was evaluated in animals inoculated with influenza A subtype H1N1 or H3N2 and followed for 42 days post inoculation (DPI). Weekly oral fluid samples (DPI 0, 7, 14, 21, 28, 35, 42) were assayed on an NP blocking ELISA (FlockChek™ Avian Influenza Virus MultiS-Screen® Antibody Test Kit, IDEXX Laboratories, Inc., Westbrook, ME) using a procedure for the detection of NP antibodies in oral fluid specimens. Because vaccination against IAV is common in the field, vaccinated groups were included for the purpose of comparing and contrasting antibody responses. The influenza antibody response measured in oral fluid specimens was statistically analyzed for the effect of treatment, time, and their interactions. In addition, the association of NP antibodies in oral fluid specimens and NP antibodies in serum was evaluated by correlation analysis and the diagnostic performance of the oral fluid assay was evaluated using receiver operating characteristic (ROC) analysis. The study was conducted under the approval (no. #11-09-6834-S) of the Iowa State University Institutional Animal Care and Use Committee and in compliance with the guidelines of the Institutional Biosafety Committee.

Animals and treatments
Animals (n = 82) in the study were obtained from one commercial farm with an inventory of approximately 600 breeding females. Piglets were weaned, ear tagged, and placed in one room of a field quarantine facility at approximately 21 days of age. Pigs were randomized to treatments (Table 1) by first assigning ear tag numbers to treatments and then blindly taking tags out of a container as the tags were applied. Serum samples were collected on -42, -21, -7, and 0 days post inoculation (DPI) and tested for IAV serum antibody to verify freedom from infection. A randomly selected subset of 28 piglets was vaccinated on DPI -42 and -21 with a multivalent, inactivated influenza vaccine (Flu-Sure® XP, Pfizer Animal Health,
Madison, NJ) following the instructions provided by the manufacturer. Piglets remained in the field quarantine facility for 33 days. On DPI -10, they were moved to the Iowa State University Livestock Infectious Disease Isolation Facility (LIDIF) and sorted into pens (Table 1). On DPI 0, pigs were intratracheally administered 2 ml of a solution containing either A/Swine/OH/511445/2007 γ H1N1 (kindly provided by Dr. Amy Vincent, USDA, ARS, NADC, Ames IA) or A/Swine/Illinois/02907/2009 Cluster IV H3N2 (kindly provided by Dr. Marie Gramer, University of Minnesota, St. Paul, MN) at a concentration of $1 \times 10^{6.5}$ tissue culture infective dose 50 (TCID<sub>50</sub>) per ml.

**Biological samples**

**Serum collection**

Blood samples ($n = 574$) were collected on DPI 0, 7, 14, 21, 28, 35, and 42 using a single-use blood collection system (Vacutainer®, Becton Dickinson, Franklin Lakes, NJ). Blood samples were centrifuged at 1800 x g for 12 min at 4°C, and then serum was aliquoted into tubes for immediate serologic testing or storage at -20°C.

**Oral fluid collection**

Oral fluid samples ($n = 182$) were collected daily from DPI -6 to 42 from each pen of pigs by suspending a length of 1/2-inch (1.3cm), 3-strand, undyed, unbleached, 100% cotton rope (Web Rigging Supply, Inc., Lake Barrington, IL) from a bracket fixed to the side of each pen. A routine was established whereby the rope was suspended in each pen for 30 minutes beginning at 7:00 a.m. Consistent with normal pig behavior (Kittawornrat and Zimmerman, 2011), the pigs chewed on the rope during this time, depositing oral fluid on the rope in the process. To extract the sample from the rope, the wet end of the rope was inserted into a plastic bag and severed from the dry portion of the rope. Then, while still within the bag, the wet rope was slowly passed through a hand wringer (BL-38, Dyna-Jet Products, Overland Park, KS), causing oral fluid to accumulate in the bottom of the bag. The bag was then pierced with a single-use needle and the contents drained into a 50 ml tube (BD Falcon centrifuge tubes®, Two Oak Park, Bedford, MA). To avoid contamination between samples, the wringer was cleaned with 70% alcohol and dried with paper towels between each use. Operators wore personal protective equipment and changed latex gloves between processing each sample. To exclude contamination between exposure groups, one individual collected oral fluid specimens from all the pens in an exposure group.
(negative control, H1N1, H3N2) and processed the ropes in the rooms in which the exposure group was housed. Immediately following collection, samples were refrigerated (4°C), processed by centrifugation at 9000 x g for 10 min at 4°C, and stored at -80°C until assayed.

**AI MultiS-Screen® Antibody Test Kit Influenza (NP ELISA)**

*Quality control* Prior to testing, the plate washer passed the dispense precision and evacuation efficiency tests and the plate reader passed the absorbance plate test, empty carrier test, and liquid test described by the manufacturer (Bio Tek® Instruments Inc., Winooski, VT). Negative and positive kit controls were run in duplicate on each plate. As described by the manufacturer, valid assays required the mean negative control OD value to be ≥ 0.60 and the mean positive control S/N ratio to be ≤ 0.50.

*Serum samples* Serum samples were tested using a blocking NP ELISA licensed by the USDA for the detection of anti-influenza NP antibodies in avian serum. This assay has also been shown to effectively detect NP antibodies in swine serum (Ciacci-Zanella et al., 2010). The NP ELISA is a blocking ELISA in which NP antibody in the sample binds to NP antigen coated to the bottom of the plate wells. In the absence of NP antibody in the sample, unbound NP antigen reacts with the kit conjugate (monoclonal antibody), which then reacts with 3, 3’, 5, 5’- tetramethyl benzidine (TMB) substrate and develops color. Thus, a lower intensity of color development reflects a greater concentration of NP antibody in the sample. Samples were tested according to the manufacturer’s instructions. Plates were read at 650 nm with an EL800 micro plate reader (Bio Tek® Instruments Inc., Winooski, VT) using GEN5™ software (Bio Tek® Instruments Inc.) Sample-to-negative (S/N) ratios were calculated for each sample according to the formula provided by the kit manufacturer (FlockChek™ Avian Influenza Virus MultiS-Screen® Antibody Test Kit, IDEXX Laboratories, Inc.). A cut-off of S/N ≤ 0.60 was used to identify antibody-positive samples.
**Oral fluid samples**  Oral fluid samples were tested using the serum blocking NP ELISA, but the assay was performed using a protocol modified to account for the lower concentration of antibody in oral fluid. Specific changes for this purpose included decreased sample dilution (undiluted vs. 1:10 in serum), increased sample volume (200 µl vs. 100 µl), and longer sample incubation (16 hr vs. one hr). To quantify assay repeatability, the entire set of oral fluid samples was tested on each of three days.

To perform the assay, undiluted oral fluid samples (200 µl) were added to wells of a 96-well plate coated with NP antigen and incubated for 16 hr at 22 °C in a refrigerated incubator. Undiluted negative and positive kit controls (100 µl) were run in duplicate and the results used to validate each plate. In-house oral fluid negative and positive reference standards (200 µl) were run in duplicate on each plate to quantify variation between plates and kit lots. In-house negative reference standards were obtained from unvaccinated, uninoculated study pigs on DPI 42. In-house positive reference standards were oral fluid samples collected over time from a barn that had tested for positive for influenza A virus using a PCR-based assay and provided three distinct levels of reactivity in the NP ELISA (Table 2). After incubation, plates were washed 3 times with 350 µl of diluted 1:10 wash solution, 100 µl of NP antibody conjugate with horseradish peroxidase (HRP) was added, and plates were incubated for 30 min. Plates were then washed 3 times with 350 µl of diluted 1:10 wash solution, 100 µl of TMB substrate was added, and plates were incubated for 15 min. Stop solution was then added to each well and plates were read immediately thereafter. The reactions were measured as optical density (OD) at wavelength of 650 nm using a plate reader. Sample-to-negative (S/N) ratios were calculated for each sample using a formula provided by the kit manufacturer.

**Statistical analysis**
Statistical analyses were performed using commercial software (SAS® Version 9.2, SAS® Institute, Inc., Cary, NC). ELISA S/N values were log₁₀ transformed to meet the distribution requirements for valid statistical analyses and analyzed by repeated measures analysis of variance (ANOVA) using pen as the subject of repeated measures. Vaccination (Y, N), inoculation (H1N1, H3N2, negative), DPI (0, 7, 14, 21, 28, 35, 42), and their interactions
were included as fixed effects, whereas day and plate were used as random effects. Fixed and random effects were analyzed using the GLIMMIX procedure in SAS®. Difference in treatment (vaccination, inoculation) groups least squares means were assessed using Tukey-Kramer’s t-test. The association of the pen level oral fluid S/N response and the mean serum S/N responses of pigs in the pen was assessed using Pearson’s correlation coefficient. \( P \)-values < 0.05 were considered statistically significant. Diagnostic sensitivity, specificity, and associated 95% confidence intervals of the NP oral fluid ELISA were estimated by receiver operating characteristic (ROC) analysis (MedCalc® 9.2.1, MedCalc Software, Mariakerke, Belgium).

RESULTS

Oral fluid collection
Although the volume of oral fluid collected was variable over time and between treatment groups (Figure 1), IAV infection had no detectable effect on the rate or volume of oral fluid collected. Among 1,196 attempted oral fluid samplings, 1,187 (99.2%) samples were collected from -3 to 42 DPI (Figure 1). The nine missing samples were from treatment groups UVH1 (1 pen on DPI 15 and 3 pens on DPI 16) and UVH3 (3 pens on DPI 4, one pen on DPI 5, and one pen on DPI 16). Among the 1,187 samples, the overall mean oral fluid volume collected was 9.6 ml ± 3.8 (mean ± SD), with a range of 0.5 to 22.5 ml. As can be seen in Figure 1, oral fluid volume increased over time concordant with the growth of the pigs.

NP blocking ELISA responses

Quality control parameters
Serum and oral fluid samples collected on DPI 0, 7, 14, 21, 28, 35, and 42 were tested using the NP ELISA (Figures 2 and 3). All plates met the manufacturer’s validity criteria, i.e., negative control OD values were \( \geq 0.60 \) and the mean S/N of the two plate positive controls was \( \leq 0.50 \) (Table 2). OD responses from in-house oral fluid negative and positive reference standards showed consistent reactions across plates (Table 2). Testing of the complete set of oral fluid samples on three separate days showed no statistical significantly differences in S/N response by testing day \( (p = 1.00) \).
**Longitudinal S/N response**

In serum samples (Figure 2), the least square mean S/N ratios of the six treatment groups at DPI 0 ranged from 0.69 to 0.86, with no significant difference in the S/N ratios among treatment groups \( p = 0.113 \). Using the cut-off of \( \leq 0.60 \), 7 of 28 vaccinated animals were serum antibody positive on the NP blocking ELISA on DPI 0. The remaining 75 pigs were NP antibody negative at DPI 0 and the 6 control pigs remained negative throughout the study. For DPI 7 through 42, the S/N response in uninoculated groups differed significantly \( p < 0.001 \) from inoculated groups. Further analysis showed that inoculation (H1N1, H3N2, negative), vaccination (Y, N), and DPI (0, 7, 14, 21, 28, 35, 42) all had significant effects on S/N ratios \( p < 0.005 \). Statistically significant two-way interactions were detected between vaccine status*DPI \( p < 0.0001 \) and inoculation*DPI \( p < 0.0001 \).

In oral fluid samples (Figure 3), the mean S/N ratios of the six treatment groups at DPI 0 ranged from 0.80 to 1.07, with no statistically significant difference in the S/N response among any of the six treatment groups \( p = 0.286 \). For DPI 7 through 42, no significant difference was detected in the S/N response between the two uninoculated groups \( \text{UVCTRL and VCTRL} \) or among the four inoculated groups \( \text{UVH1, V_H1, UVH3, and V_H3} \). For the same period, the S/N response in uninoculated groups \( \text{UVCTRL and VCTRL} \) differed significantly \( p < 0.001 \) from inoculated groups \( \text{UVH1, V_H1, UVH3, and V_H3} \). On DPI 42, the last day of observation, the S/N ratio in vaccinated groups increased (became less positive), but the S/N ratios of all inoculated groups remained significantly different from uninoculated groups \( p < 0.05 \). Further analysis showed that inoculation (H1N1, H3N2, negative), and DPI (0, 7, 14, 21, 28, 35, 42) had significant effects on S/N values \( p < 0.0001 \), but vaccination did not \( p = 0.534 \). Statistically significant two-way interactions were detected between vaccine status*DPI \( p = 0.011 \) and inoculation*DPI \( p < 0.0001 \).

**Serum vs. oral fluid responses**

For the two uninoculated groups \( \text{UVCTRL and VCTRL} \), the overall mean serum and oral fluid S/N ratios for DPI 7 to 42 were 0.81 ± 0.13 (mean ± SD) and 1.05 ± 0.08 (mean ± SD). Among the four inoculated groups \( \text{UVH1, V_H1, UVH3, and V_H3} \), the overall means for the same period were 0.35 ± 0.16 (mean ± SD) in serum and 0.46 ± 0.09 (mean ± SD) in oral fluid. Thus, S/N responses in oral fluid samples trended...
higher than serum S/N responses. A pen-level evaluation of the association between the pen level oral fluid S/N response and the mean serum S/N responses of pigs within the pen found a statistically significant association ($p < 0.0001$) between serum and oral fluid S/N responses and a Pearson’s correlation coefficient of 0.796 (Figure 4).

**Evaluation of test performance**

The diagnostic performance of the NP blocking ELISA was evaluated by receiver operating characteristic (ROC) curve analysis using the S/N results from 182 oral fluid samples. This analysis produced an estimate of the area under the ROC curve (AUC) of 0.994 and the cut-off values and associated sensitivity and specificity shown in Table 3 and Figure 5.

**DISCUSSION**

Influenza A virus is an important component of the porcine respiratory disease complex (PRDC) (Thacker et al., 2001) and a pathogen with major economic impacts on swine production. Thus, Holtkamp (2007a, b) reported that influenza was among the three most costly infectious diseases in every stage of pig production in the United States, i.e., breeding, nursery, and finishing. But whether the focus is swine production or public health, the challenge with IAV in swine populations is collecting a sufficient number of samples at the right time in the course of the infection to accurately describe the epidemic or endemic situation. The detection of IAV has historically been based on testing individual pig nasal swab (virus detection) or serum (antibody detection) specimens. While individual pig sampling is adequate for the diagnosis of clinical IAV infections, the collection of adequate numbers of individual pig samples is too costly and labor-intensive for routine influenza surveillance or large-scale ecological studies.

The timing of sampling is critically important because negative PCR or virus isolation results may reflect the transient nature of the infection rather than recent events or the true status of the population. Antibody provides a longer record of IAV infection, but the diagnostic performance of the current serum antibody assays, i.e., hemagglutination-inhibition (HI) or influenza subtype-specific ELISAs, has been problematic. The HI response, based on the
binding of hemagglutinin (HA) protein on the viral surface and sialic acid receptors on erythrocytes, is affected by a number of factors, including dependence of the HI response on the degree of homology between the virus that stimulated IAV antibody production in the pig and the virus strain in the test (Barbé et al., 2009; Van Reeth et al., 2006), interference of antibodies against other IAV subtypes (Long et al., 2004), nonspecific inhibitors of IAV in swine serum, such as carbohydrate and sialic acid (Ryan et al., 1991), differences in the ability of IAVs to agglutinate erythrocytes (Levy and Wager, 1958), and differences in erythrocyte agglutinating properties among species and even among individual animals within species (Long et al., 2004; Schmidt and Lennette, 1970). Individually or in combination, these variables can markedly affect HI serum antibody titers. Importantly, deviations from the optimized combination of factors tend to produce false-negative results (Julkunen et al., 1985; Rossow et al., 2003). Similarly, the current commercial swine H1N1 and H3N2 ELISAs (IDEXX Laboratories, Inc.) possess low diagnostic sensitivity; presumably the result of differences between the antigen present in the assay and the viruses currently circulating in swine populations (Barbé et al., 2009; Long et al., 2004; Lorusso et al., 2011; Van Reeth et al., 2006; Yoon et al., 2004).

As reviewed elsewhere (Prickett and Zimmerman, 2010), oral fluid samples have been shown to be a viable diagnostic specimen for the detection of a variety of pathogens and/or antibodies in a variety of species. Pertinent to IAV, contemporary work under both experimental and field settings has shown that the virus can be detected in porcine oral fluid specimens using PCR-based assays (Detmer et al., 2011; Ramirez et al., 2012; Romagosa et al., 2011). The current study was based on the detection of IAV NP antibody in porcine oral fluid specimens. Nucleoprotein is a multifunctional protein associated with viral transcription, replication, and intracellular trafficking of the viral genome (Noda et al., 2006; Potela and Digard, 2002; Ye et al., 2006). NP is highly conserved among IAVs and infection with any influenza subtype induces IAV-specific NP antibodies (de Boer et al., 1990; El Hefnawi et al., 2011; Wu et al., 2007). This universal antibody response has tangible diagnostic benefits, e.g., a non-commercial NP blocking ELISA detected serum antibodies induced by IAV infections in humans, ferrets, swine, horses, chickens, ducks, guinea pigs, mice, and seals (de Boer et al., 1990). The present study expanded upon earlier work by
demonstrating that porcine oral fluid specimens also contain diagnostically relevant levels of NP antibody, with no detectable difference between subtypes H1N1 or H3N2 in diagnostic sensitivity and specificity.

These results are also in general agreement with reports of the detection of IAV antibodies in oral fluid specimens collected from humans (Brokstad et al., 1995a, b). In humans, the presence of IAV antibodies in individual oral fluid specimens, as measured by an indirect ELISA, peaked at 8 to 9 days post vaccination (DPV) and declined thereafter (Brokstad et al., 1995a). The majority of IAV antibody through DPV 45 was IgA (Brokstad et al., 1995a), especially subclass IgA1 (Brokstad et al., 1995b). In swine, there are no reports describing the kinetics of IAV antibodies in oral fluids, but the kinetics of both IgA and IgG have been reported in nasal lavage and bronchoalveolar lavage (BAL) specimens following inoculation with IAV subtypes H1N1 (Larsen et al., 2000) and H3N2 (Heinen et al., 2000). Using a homologous indirect ELISA, IgA was determined to be the predominant antibody isotype in both sample types. In BAL, IgA peaked at day 14 post inoculation and IgG peaked at day 21 (Larsen et al. 2000). Similar results for nasal lavage and BAL samples were reported in pigs infected with an H3N2 isolate. Heinen et al. (2000) detected NP IgM and IgA using an antibody capture ELISA and IgG1 using an indirect double antibody sandwich assay. In BAL, IgA was the predominant immunoglobulin. Both IgA and IgG1 peaked at day 15, while IgM peaked at 7 days post inoculation (Heinen et al., 2000).

Improved methods to surveil IAV infections in swine populations should continue to be of importance to swine producers and veterinarians because of IAV's zoonotic potential, disregard for inter-species barriers, and the public's view of the role swine play in the dispersion of the virus. The results of this study support the conclusion that pen-based oral fluids could serve a role in the surveillance of IAV in swine populations by facilitating the collection and testing of large sample numbers in a welfare-friendly, worker-friendly, cost-effective manner.
ACKNOWLEDGEMENTS

This work was supported in part by Pork Checkoff funds distributed through the National Pork Board (#09-193), Pfizer® Animal Health, and IDEXX Laboratories, Inc. As noted, authors A. Ballagi and S. Lizano are employees of IDEXX Laboratories, Inc. The remaining author(s) declare that they had no conflicts of interest with respect to their authorship and/or the publication of this manuscript.

REFERENCES


Table 1. Experimental design

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Inoculant</th>
<th>Vaccination*</th>
<th>No. of pigs</th>
<th>No. of pens</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unvaccinated control (UV\textsubscript{CTRL})</td>
<td>None</td>
<td>No</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>Vaccinated control (V\textsubscript{CTRL})</td>
<td>None</td>
<td>Yes</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>Unvaccinated H1N1 (UV\textsubscript{H1})</td>
<td>H1N1</td>
<td>No</td>
<td>24</td>
<td>8</td>
</tr>
<tr>
<td>Vaccinated H1N1 (V\textsubscript{H1})</td>
<td>H1N1</td>
<td>Yes</td>
<td>11</td>
<td>3</td>
</tr>
<tr>
<td>Unvaccinated H3N2 (UV\textsubscript{H3})</td>
<td>H3N2</td>
<td>No</td>
<td>24</td>
<td>8</td>
</tr>
<tr>
<td>Vaccinated H3N2 (V\textsubscript{H3})</td>
<td>H3N2</td>
<td>Yes</td>
<td>11</td>
<td>3</td>
</tr>
</tbody>
</table>

*Vaccinated on DPI -42 and -21 with a multivalent, inactivated influenza vaccine (Flu-Sure\textsuperscript{®} XP, Pfizer Animal Health, Madison, NJ, USA).
<table>
<thead>
<tr>
<th>Controls</th>
<th>Mean</th>
<th>Range</th>
<th>Standard Deviation</th>
<th>95% Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kit antibody negative control (serum)</td>
<td>1.47</td>
<td>1.29 - 1.67</td>
<td>0.13</td>
<td>1.40, 1.53</td>
</tr>
<tr>
<td>Kit antibody positive control (serum)</td>
<td>0.38</td>
<td>0.28 - 0.49</td>
<td>0.06</td>
<td>0.34, 0.41</td>
</tr>
<tr>
<td>In-house antibody negative control (oral fluid)</td>
<td>1.53</td>
<td>1.30 - 1.71</td>
<td>0.11</td>
<td>1.48, 1.58</td>
</tr>
<tr>
<td>In-house low antibody positive control (oral fluid)</td>
<td>0.82</td>
<td>0.72 - 0.93</td>
<td>0.06</td>
<td>0.79, 0.85</td>
</tr>
<tr>
<td>In-house moderate antibody positive control (oral fluid)</td>
<td>0.43</td>
<td>0.37 - 0.48</td>
<td>0.04</td>
<td>0.41, 0.45</td>
</tr>
<tr>
<td>In-house high antibody positive control (oral fluid)</td>
<td>0.24</td>
<td>0.20 - 0.28</td>
<td>0.03</td>
<td>0.22, 0.25</td>
</tr>
</tbody>
</table>

*FlockChek® Avian Influenza MultiS-Screen Antibody Test Kit, IDEXX Laboratories, Inc., Westbrook, Maine USA*
Table 3.  S/N (sample/negative) cut-off and associated diagnostic sensitivity and specificity from a commercial influenza ELISA* performed using a protocol modified to detect influenza virus nucleoprotein antibodies in swine oral fluids

<table>
<thead>
<tr>
<th>S/N Cut-off</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤ 0.87</td>
<td>100.0 (98.6, 100.0)</td>
<td>79.4 (71.2, 86.1)</td>
</tr>
<tr>
<td>≤ 0.85</td>
<td>99.6 (97.9, 99.9)</td>
<td>84.1 (76.6, 90.0)</td>
</tr>
<tr>
<td>≤ 0.80</td>
<td>99.2 (97.3, 99.9)</td>
<td>88.1 (88.1-93.2)</td>
</tr>
<tr>
<td>≤ 0.75</td>
<td>98.1 (95.6, 99.4)</td>
<td>92.1 (85.9, 96.1)</td>
</tr>
<tr>
<td>≤ 0.70</td>
<td>97.0 (94.1, 98.7)</td>
<td>93.7 (87.9, 97.2)</td>
</tr>
<tr>
<td>≤ 0.65</td>
<td>95.1 (91.7, 96.5)</td>
<td>96.8 (92.1, 99.1)</td>
</tr>
<tr>
<td>≤ 0.60</td>
<td>90.5 (86.3, 97.3)</td>
<td>96.0 (91.0, 98.7)</td>
</tr>
<tr>
<td>≤ 0.55</td>
<td>86.0 (81.2, 89.9)</td>
<td>99.2 (95.6, 99.9)</td>
</tr>
<tr>
<td>≤ 0.52</td>
<td>81.1 (75.8, 85.6)</td>
<td>100.0 (97.1, 100.0)</td>
</tr>
</tbody>
</table>

*FlockChek® Avian Influenza MultiS-Screen Antibody Test Kit, IDEXX Laboratories, Inc., Westbrook, Maine USA
Figure 1. Oral fluid volume collected from day post inoculation -3 to 42
Figure 2. Influenza virus serum antibody response by treatment group as measured using a commercial nucleoprotein blocking ELISA (mean ± SE)
Figure 3. Influenza virus oral fluid antibody response by treatment group as measured using a commercial nucleoprotein blocking ELISA (mean ± SE)
Figure 4. Correlation of ELISA sample-to-negative (S/N) responses between serum and oral fluid specimens
Figure 5. Diagnostic performance of commercial nucleoprotein blocking ELISA adapted to oral fluid specimens as a function of sample-to-negative (S/N) cut off
CHAPTER 4. KINETICS OF INFLUENZA A VIRUS NUCLEOPROTEIN ANTIBODY (IgM, IgA, AND IgG) IN SERUM AND ORAL FLUID SPECIMENS FROM PIGS INFECTED UNDER EXPERIMENTAL CONDITIONS


Accepted for publication in Vaccine

ABSTRACT

Indirect influenza A virus (IAV) nucleoprotein (NP) antibody ELISAs were used to compare the kinetics of the NP IgM, IgA, and IgG responses in serum and pen-based oral fluid samples collected from 82 pigs followed for 42 days post inoculation (DPI). Treatment categories included vaccination (Y/N) and inoculation (Y/N) with contemporary H1N1 or H3N2 isolates. Antibody ontogeny was markedly affected by vaccination status, but no significant differences were detected between H1N1 and H3N2 inoculated groups of the same vaccination status (Y/N) in IgM, IgA, or IgG responses. Therefore, these data were combined in subsequent analyses. The correlation between serum and oral fluid responses was evaluated using the pen-based oral fluid S/P response versus the mean serum S/P response of pigs within the pen. IgM responses in serum and oral fluid were highly correlated in unvaccinated groups (r = 0.810), as were serum and oral fluid IgG responses in both unvaccinated (r = 0.839) and vaccinated (r = 0.856) groups. In contrast, the correlation between serum and oral fluid IgA was weak (r ~ 0.3), regardless of vaccination status. In general, vaccinated animals exhibited a suppressed IgM response and accelerated IgG response. The results from this study demonstrated that NP-specific IgM, IgA, and IgG antibody were detectable in serum and oral fluid and their ontogeny was influenced by vaccination status, the time course of the infection, and specimen type.
INTRODUCTION

Influenza A virus (IAV) is capable of infecting a wide variety of avian and mammalian species, including swine. In swine, IAV infection may be subclinical or present acute respiratory or reproductive signs, e.g., stillborn piglets (Wesley et al., 2004) or abortion (Vannier et al., 1999). In the field, respiratory signs and/or mortality rates are commonly exacerbated by the presence of common respiratory pathogens, e.g., porcine reproductive and respiratory syndrome virus (PRRSV), porcine circovirus type 2 (PCV-2), Mycoplasma hyopneumoniae, Actinobacillus pleuropneumoniae, and Bordetella bronchiseptica (Bochev et al., 2007; Choi et al., 2003). Formerly considered a seasonal infection, IAV infection in contemporary swine herds circulates in all age groups throughout the year (Choi et al., 2003; Simon-Grifé et al., 2012), including piglets prior to weaning (Corzo et al., 2012).

IAV is an enveloped RNA virus with a segmented, negative sense, single-stranded genome which, typical of RNA viruses, exhibits rapid genetic evolution via point mutations and genetic reassortment (van Reeth et al., 2012). Influenza A virus subtype classification is based on the presence of two external surface proteins, hemagglutinin (HA) and neuraminidase (NA). To date, 17 HAs and 10 NAs have been identified (Tong et al., 2012; Zhu et al., 2012). Subtypes H1N1, H3N2 and H1N2 are common in pig populations around the world (Brown et al., 2000; Choi et al., 2002), but other subtypes are occasionally reported, e.g., H1N7 in England (Brown et al., 1994); H3N1 in the U.S. and Korea (Lekcharoensuk et al., 2006; Shin et al., 2006); H2N3 in the U.S. (Ma et al., 2007); H5N2 in Korea (Lee et al., 2009); H4N6 in Canada (Karasin et al., 2000); H7N2 in Korea (Kwon et al., 2011); and H9N2 in China (Xu et al., 2004).
Historically, IAV serology has relied on the hemagglutinin inhibition (HI) assay, which is based on the ability of anti-IAV antibodies to block the virus from agglutinating red blood cells. However, the performance of the HI assay is affected by the interaction of a number of biological factors, including the degree of homology between the virus that stimulated IAV antibody production in the pig and the virus strain used in the HI test (Barbé et al., 2009; Van Reeth et al., 2006). In particular, failure to achieve the optimal combination of factors tends to result in false-negatives (Julkunen et al., 1985; Rossow et al., 2003).

Given that the IAVs circulating in swine populations are constantly evolving, a universal assay capable of detecting antibodies against any IAV subtype would be desirable. Among the possibilities, nucleoprotein (NP) is considered a good candidate because it is a highly conserved internal protein (De Boer et al., 1990). Previously it was shown that an NP blocking ELISA provided for the detection of IAV antibody in swine regardless of subtype (Ciacci-Zanella et al, 2010). Later work expanded on the initial observations in pigs by showing that NP antibody in serum correlated with the detection of NP antibody in oral fluids (Panyasing et al., 2012). The purpose of the present study was to expand on the earlier reports by describing the ontogeny of the NP-specific IgM, IgA, and IgG responses in serum and oral fluids from vaccinated and non-vaccinated pigs inoculated with IAV H1N1 or H3N2.

MATERIALS AND METHODS

Experimental Design
Immunoglobulin M (IgM), A (IgA), and G (IgG) responses against influenza A virus (IAV) nucleoprotein (NP) were evaluated in serum and oral fluid specimens collected for 42 days post inoculation (DPI) from pigs [unvaccinated (UV) or vaccinated (V)] inoculated with IAV subtypes H1N1 or H3N2. Serum and oral fluid NP antibody (IgM, IgA, IgG) kinetics were analyzed in the context of vaccination status (Y/N), inoculation status (Y/N), IAV inoculum (H1N1 or H3N2), time post-inoculation, and their interactions. The ability of the serum and oral fluid NP ELISAs to discriminate between negative and inoculated pigs was evaluated using receiver operating characteristic (ROC) analyses.
**Animals and treatments**

The study was conducted with the approval of the Iowa State University Office for Responsible Research (#11-09-6834-S). Animals \((n=82)\) in the study were obtained from one commercial farm with an inventory of approximately 600 breeding females. Piglets were weaned, ear tagged, and placed in one room of a field quarantine facility at approximately 21 days of age (DPI -43). Pigs were randomized to one of six treatments by first assigning ear tag numbers to treatments and then blindly taking tags from a container as the tags were applied. To verify freedom from IAV infection, serum samples were collected on \(-42, -21, -7,\) and \(0\) days post inoculation (DPI) and tested for IAV serum antibody using a commercial NP blocking ELISA (IDEXX Influenza A Ab Test, IDEXX Laboratories, Inc., Westbrook, ME). A randomly selected subset of 28 piglets was vaccinated on DPI -42 and -21 with a multivalent, inactivated influenza vaccine (Flusure®XP, Pfizer Animal Health, Madison, NJ).

On DPI -10, animals were moved to the Iowa State University Livestock Infectious Disease Isolation Facility (LIDIF) and placed in treatment groups: (1) unvaccinated, uninoculated controls \((UV_{CTRL}, 2\) pens of 3 pigs); (2) vaccinated, uninoculated controls \((V_{CTRL}, 2\) pens of 3 pigs); (3) unvaccinated, inoculated with H1N1 \((UV_{H1}, 8\) pens of 3 pigs); (4) vaccinated, inoculated with H1N1 \((V_{H1}, 3\) pens of 3 or 4 pigs); (5) unvaccinated, inoculated with H3N2 \((UV_{H3}, 8\) pens of 3 pigs); and (6) vaccinated, inoculated with H3N2 \((V_{H3}, 3\) pens of 3 or 4 pigs).

On DPI 0, pigs were intratracheally administered 2 ml of a solution containing either A/Swine/OH/511445/2007 \(\gamma\) H1N1 (kindly provided by Dr. Amy Vincent, USDA, ARS, NADC, Ames IA) or A/Swine/Illinois/02907/2009 Cluster IV H3N2 (kindly provided by Dr. Marie Gramer, University of Minnesota, St. Paul, MN) at a concentration of \(1 \times 10^{6.5}\) tissue culture infective dose 50 (TCID_{50}) per ml, as designated for treatment groups.

**Biological samples**

Serum samples were collected from 82 pigs on DPIs -42, -21, -7, 0, 7, 14, 21, 28, 35, and 42 for a total of 819 samples. Serum samples were processed by centrifugation at 1500 x g for 12 min at 4°C, after which serum was aliquoted into tubes and stored at -20°C. Pen-based
oral fluid samples were collected from 26 pens daily from DPI 0 to 42 for a total of 510 samples. Oral fluid samples were processed by centrifugation at 9000 x g for 10 min at 4°C, after which samples were aliquoted into tubes and stored at -80°C.

**Serum NP antibody isotype-specific indirect ELISAs**

Serum samples were assayed for IAV-specific antibody isotypes using plates and reagents, i.e., sample diluent, substrate, stop solution, and wash solution, from a commercial NP blocking ELISA kit (IDEXX Influenza A Ab Test). The manufacturer's protocol was modified, as described below, to detect IgM, IgA, or IgG.

**Serum ELISA plate controls** Negative and isotype-specific positive control sera were run in duplicate on each plate. Controls were used to validate plate performance and to calculate sample-to-positive (S/P) ratios. The negative control consisted of a pool of serum collected on DPI 42 from the 6 negative control (UVCTRL) pigs diluted 1:100 (IgM), 1:50 (IgA) and 1:800 (IgG) with sample diluent. Positive controls for serum IgM, IgA, and IgG assays were obtained from pooled serum collected on DPI 7 (IgM), 14 (IgA), and 42 (IgG) from the 24 unvaccinated pigs inoculated with IAV subtype H1N1 (UVH1). Positive control sera were diluted 1:100 (IgM), 1:10 (IgA), and 1:400 (IgG) with sample diluent for isotype-specific assays. Optical density (OD) values of negative control sera for IgM-, IgA-, and IgG-specific ELISA assays were 0.2, 0.2, and 0.4 respectively; whereas OD values of positive control sera for IgM, IgA and IgG assays were 0.8, 0.8, and 1.0. Controls were prepared for use, aliquoted into 2 ml cryovials, and stored at -20 °C.

**Preparation of conjugates** The appropriate dilution of horseradish peroxidase (HRP)-conjugated goat anti-pig IgM (A100-100P, Bethyl laboratories Inc., Montgomery, TX), IgA (A100-102P, Bethyl laboratories Inc.), or IgGFC (A100-104P, Bethyl laboratories, Inc.) was calculated for each lot of ELISA plates to standardize the strength of the reaction and ensure the reproducibility of results. This was achieved by determining the dilution of anti-pig IgM, IgA, or IgG conjugate that matched the OD values of negative and positive controls for each assay (described in the previous paragraph). Conjugate concentrations were prepared using a
commercial conjugate diluent (IDEXX PRRS X3 Ab Test, IDEXX Laboratories, Westbrook, ME) and stirred for 48 hours at 4°C before use.

**Serum NP antibody isotype-specific protocols** For the detection of IgM antibody, serum samples were diluted 1:50 (5 µl of sample with 245 µl of sample diluent), after which plates were loaded with 100 µl of diluted samples and pre-diluted (1:100) negative and positive controls (in duplicate). Plates were incubated at 37°C for 2h and then washed 3 times with 400 µl of ELISA kit wash solution diluted 1:10 with deionized water. Next, 100 µl of HRP-conjugated goat anti-pig IgM antibody diluted 1:8,000 was added to each well and plates incubated at 37°C for 1h. Plates were washed 3 times with 400 µl of wash solution, and 100 µl of 3, 3’, 5, 5’-tetramethyl benzidine (TMB) substrate was added. Plates were incubated at room temperature for 15 min, 100 µl of stop solution were added to stop the reaction, and plates were read immediately thereafter. Reactions were measured as OD value at a wavelength of 650 nm using an ELISA plate reader (Biotek® Instruments Inc., Winooski, VT) operated with commercial software (GEN5™, Biotek® Instruments Inc.)

The indirect ELISA protocol used for the detection of serum NP-specific IgG and IgA followed the IgM protocol with minor exceptions. Specifically, serum samples for the IgG assay were diluted 1:50 (5 µl of a sample with 245 µl of sample diluent) and HRP-conjugated goat anti-pig IgGFC antibody was diluted 1:10,000; whereas serum samples for the IgA assay were diluted 1:10 (20 µl of a sample with 180 µl of sample diluent) and HRP-conjugated goat anti-pig IgA was diluted 1:2,000.

**Oral fluid NP antibody isotype-specific indirect ELISAs**

**Preparation of ELISA plates** Oral fluid samples were assayed on ELISA plates manually coated with commercially produced NP antigen derived from a human IAV isolate (A/Puerto Rico/8/34/Mount Sinai (H1N1) segment 5) cloned into a baculovirus expression system (Cat no. IMR-274, Imgenex© Corporation, San Diego, CA). In brief, 96-well microtitration plates (Cat no. 446469, Fisher Scientific, Hanover, Illinois USA) were coated with one µg of NP antigen (Imgenex© Corporation) per well by adding 100 µl of a 1:100 dilution of NP antigen in 0.05 M carbonate-bicarbonate buffer (C3041, Sigma-Aldrich, St.
Coated plates were then incubated at 25°C overnight. After incubation, plates were washed 3 times with 400 µl of ELISA kit wash solution diluted 1:10 with deionized water. Next, 300 µl of 1% bovine serum albumin (BSA) blocking buffer [50 g of sucrose (Sigma-Aldrich), 2.5 g of dextran (Sigma-Aldrich), 25 ml of glycerol (Sigma-Aldrich), 500 µl of proline (Sigma-Aldrich), 5 g of BSA (Cat no. 001-000-162 Jackson, ImmunoResearch Laboratories, West Grove, PA) plus 1X PBS (Sigma-Aldrich) to total 0.5 liter] were added and incubated at 25°C for 2 h. The blocking buffer was removed without washing. Plates were then dried at 37°C for 2 h and stored at 4°C in a seal bag with desiccant packs until used. To evaluate the consistency of the coating process, each lot of NP-coated plates was evaluated by testing one well of each plate with one antibody-positive sample and calculating the coefficient of variation (standard deviation:mean) of the OD responses. Plate lots with a coefficient of variation ≥10% were rejected.

**Preparation of conjugates** Secondary antibody conjugates for the oral fluid NP antibody isotype-specific indirect ELISAs were prepared as described for the serum ELISAs.

**Oral fluid ELISA plate controls** Negative and positive controls were run in duplicate on each plate. The negative control consisted of a pool of serum collected on DPI 42 from the 6 negative control (UVCTRL) pigs diluted 1:400, 1:10, and 1:200 with ELISA kit sample diluent for IgM, IgA, and IgG assays, respectively. The OD values of negative controls were 0.2, 0.2, and 0.3 for oral fluid IgM, IgA, and IgG assays. Positive controls for oral fluid assays were obtained from pooled serum collected on DPI 7 (IgM), 14 (IgA), and 42 (IgG) from the 24 unvaccinated pigs inoculated with IAV subtype H1N1 (UVH1) and diluted 1:300 (IgM), 1:5 (IgA), and 1:600 (IgG) with ELISA kit sample diluent for the isotype-specific ELISAs. The OD values of the positive controls oral fluid for IgM, IgA, and IgG assays were 1.2, 1.4, and 1.2, respectively. Controls were prepared for use, aliquoted into 2 ml cryovials, and stored at -20 °C.

**Oral fluid NP antibody isotype-specific protocols** The oral fluid NP antibody isotype-specific assay protocols used the same sample dilution, sample volume, incubation time, and incubation temperature, but differed in conjugate concentrations. The reagents used in the
assays, i.e., substrate, stop solution, and wash solution, were from a commercial NP blocking ELISA kit (IDEXX Influenza A Ab Test). To perform the assays, 200 µl of undiluted oral fluid specimens and 100 µl of pre-diluted negative and positive controls (in duplicate) were added to the wells of an NP-coated plate and incubated at 4°C for 16 h. After incubation, plates were washed 3 times with 400 µl of ELISA kit wash solution diluted 1:10 with deionized water. For the detection of IgM antibody, 100 µl of HRP-conjugated goat anti-pig IgM antibody diluted 1:2,000 was added to each well. For the detection of IgA or IgG antibody, HRP-conjugated goat anti-pig IgA antibody diluted 1:2000 or HRP-conjugate goat anti-pig IgGFC antibody diluted 1:1,500 was used. After adding conjugate, plates were incubated at 37°C for 1 h. Plates were then washed 3 times with 400 µl of wash solution, 100 µl of TMB substrate was added, and plates were incubated at room temperature for 15 min. 100 µl of stop solution was then added and plates were read immediately thereafter. Reactions were measured as OD value at a wavelength of 650 nm using an ELISA plate reader (Biotek® Instruments Inc) operated with commercial software (GEN5™, Biotek® Instruments Inc.).

**S/P ratio calculation**

Serum and oral fluid IgM, IgA, and IgG responses were represented as sample-to-positive (S/P) ratios calculated using the following formula.

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

**Analysis**

The effects of treatment (vaccination and inoculation status) on antibody isotype-specific S/P responses over time were analyzed by repeated measures analysis of variance (ANOVA) using NP ELISA S/P ratios from weekly serum samples and oral fluid samples collected on DPI 0 to 14, 17, 21, 28, 35, and 42 (SAS® Institute, Inc., Cary, NC). Differences in treatment group least squares means were assessed using the Tukey-Kramer test. The association of the pen-level oral fluid S/P response and the mean of the serum S/P responses
of pigs in the pen was assessed using Pearson’s correlation coefficient. $P$-values < 0.05 were considered statistically significant.

The performance of serum and oral fluid NP isotype-specific ELISAs was evaluated by first establishing optimum S/P cut-offs and then calculating the percent positives within treatment groups over time. The optimum cut-off was determined using receiver operating characteristic (ROC) analysis (MedCalc® Version 12.2.1.0, MedCalc Software, Mariakerke, Belgium). Known negatives were restricted to group UVCTRL (DPI 0 to 42) and known positives to group UVH1H3. Within group UVH1H3, serum IgM positives were defined as samples from DPI 7; IgA positives as samples from DPI 14, and IgG positives as samples from DPIs 14, 21, 28, 35, and 42. Oral fluid positives were from group UVH1H3 on DPIs 5 through 11 (IgM), DPIs 6 through 14, 17, 21, 28, 35, and 42 (IgA), and DPIs 8 through 14, 17, 21, 28, 35, and 42 (IgG).

RESULTS

To verify freedom from IAV infection prior to inoculation, serum samples collected on DPI -42, -21, -7, and 0 days were tested for IAV serum antibody using a commercial NP blocking ELISA (IDEXX Influenza A Ab Test, IDEXX Laboratories, Inc.). No NP-specific serum antibody was detected at DPI -42, -21, -7 or 0 in any of the 54 unvaccinated pigs. Among vaccinated pigs, 4 of 28 and 9 of 28 vaccinated pigs were NP antibody positive at DPI -7 and 0, respectively (data not shown).

Quantitative responses of NP isotype-specific antibody ELISAs

*NP-specific serum antibody* Quantitative IgM, IgA, and IgG responses (S/P ratios) in serum by time post inoculation are given in Figure 1. Within vaccination status (UV or V), repeated measures ANOVA found no significant differences ($p < 0.05$) over time in IgM, IgA, and IgG responses between H1N1 and H3N2 inoculated groups. Therefore, the results were combined into groups UVH1H3 and VH1H3 for the remainder of the analyses.
The NP-specific IgM S/P ratios gradually increased over time in all groups prior to inoculation (DPI -42 to 0). Compared to DPI -42, IgM S/P ratios were significantly greater at DPI -21, -7 and 0 in all treatment groups \( (p < 0.0001) \). Following inoculation, an NP-specific IgM antibody response was only detected in group UV_{H1H3}. This response was significantly different from groups UV_{CTRL}, V_{CTRL}, and V_{H1H3} at DPI 7 \( (p < 0.0001) \).

Similar to IgM, the NP-specific IgA S/P ratios gradually increased over time in all groups prior to inoculation (DPI -42 to 0). Following inoculation, no statistically significant difference in the NP-specific IgA antibody was detected among the 4 treatment groups (UV_{CTRL}, V_{CTRL}, UV_{H1H3}, and V_{H1H3}), except at DPI 14 when the IgA response in group UV_{H1H3} was significantly greater than UV_{CTRL}, V_{CTRL}, and V_{H1H3} (Figure 1).

In contrast to IgM and IgA, no NP-specific IgG S/P response was detected in unvaccinated groups (UV_{CTRL} and UV_{H1H3}) prior to inoculation (DPI -42 to 0). Following inoculation, the IgG response in group UV_{H1H3} was greater than UV_{CTRL} at DPI 14 \( (p < 0.0001) \), achieving its maximum response at DPI 35 (Figure 1). Vaccination produced an NP-specific serum S/P IgG response in vaccinates at DPls -7 and 0 (Figure 1) that differed from unvaccinated pigs \( (p < 0.0015) \). The mean IgG S/P ratio in V_{CTRL} trended to decline after DPI -7, but remained greater than UV_{CTRL} \( (p < 0.05) \) through DPI 35. In V_{H1H3}, the IgG response reached a maximum response at DPI 14 and remained stable through the end of study. A comparison of V_{H1H3} and UV_{H1H3} found no differences between these groups from DPI 21 to 42.

**NP-specific oral fluid antibody** Quantitative IgM, IgA, and IgG responses in oral fluid by time post inoculation are given in Figure 2. Within vaccination status (UV or V), repeated measures ANOVA found no significant differences over time in IgM, IgA, and IgG responses between H1N1 and H3N2 inoculated groups. Therefore, the results were combined into groups UV_{H1H3} and V_{H1H3} for the remainder of the analyses.

The NP-specific IgM S/P ratio in UV_{H1H3} was significantly different \( (p < 0.01) \) from groups UV_{CTRL}, V_{CTRL}, and V_{H1H3} at DPI 5 to 11 \( (p < 0.05) \). The IgM response in UV_{H1H3} peaked at DPI 8, after which it rapidly declined. The analysis found that the IgA S/P ratios in groups UV_{H1H3} and V_{H1H3} were significantly different \( (p < 0.001) \) from groups UV_{CTRL} and V_{CTRL} on
DPI 6 and remained so through DPI 42. The NP-specific IgG S/P responses in UVH1H3 and VH1H3 were significantly different from groups UVCTRL and VCTRL at DPI 8 (p < 0.001) and remained so through DPI 42. The maximum IgG response in both UVH1H3 and VH1H3 occurred at DPI 10.

**Qualitative responses of NP isotype-specific antibody ELISAs**

ROC analyses of IgM, IgA, and IgG NP ELISAs established the optimum S/P cut-offs for serum at 0.84 (IgM), 0.75 (IgA) and 0.60 (IgG) and for oral fluid at 0.50 (IgM), 0.60 (IgA), and 0.60 (IgG). These cut-offs were used to calculate the percent of serum and oral fluid positive samples by DPI, as shown in Tables 1 and 2.

**NP-specific serum antibody**

Known negative serum samples included 60 samples collected between DPI -42 and 42 from the 6 pigs in group UVCTRL and 192 serum samples collected between DPI -42 to 0 from the 48 pigs in group UVH1H3. Based on the cut-offs established in the ROC analysis, 98.4%, 97.2% and 100% of these samples were ELISA negative for IgM, IgA, and IgG, respectively. Specifically, the IgM ELISA produced 4 false positive results in UVH1H3 (2 each on DPI -7 and 0). The IgA ELISA produced 2 false positive results in UVCTRL (one each on DPIs 28 and 42) and 5 false positive results in UVH1H3 (2 on DPI-7 and 3 on DPI 0).

The proportion of ELISA serum antibody-positive pigs varied by DPI, treatment group, and antibody isotype. A small number of IgM and/or IgA, seropositive animals were detected among vaccinates (VCTRL and VH1H3) on DPI -21, -7, and 0, i.e., prior to IAV inoculation. In contrast, vaccination produced a detectable NP-specific serum IgG response in ≥40% of vaccinates at DPIs -7 and 0 (Table 1).

Following inoculation, the IgM and IgA serum responses in group UVH1H3 were both more robust and of longer duration than VH1H3, but in neither case was a response detected in all animals (Table 1). That is, 47 of 48 serum samples from UVH1H3 were IgM positive whereas only 1 of 22 from VH1H3 were positive at DPI 7. Similarly, 24 of 28 were IgA positive in group UVH1H3 whereas only 4 of 22 were positive in group VH1H3 at DPI 14. In contrast, IgG
was detected in 100% of pigs in group \(V_{HIH3}\) by DPI 7 and 100% of pigs in group \(UV_{HIH3}\) by DPI 14 (Table 1) and both groups remained positive through DPI 42.

**NP-specific oral fluid antibody** Known negative oral fluid samples included 40 oral fluid samples collected between 0 and 42 from the 2 pens in group \(UV_{CTRL}\). All of these samples were ELISA negative for IgM, IgA, and IgG (Table 2). Likewise, no NP-specific IgM, IgA or IgG was detected in the 40 oral fluid samples collected from group \(V_{CTRL}\) between DPI 0 through 42. However, one of six samples from group \(V_{HIH3}\) was positive for IgM on DPI 0, although these 6 samples were negative for IgA and IgG.

Following inoculation, the IgM response in \(UV_{HIH3}\) was more robust and of longer duration than \(V_{HIH3}\) (Table 2). In \(UV_{HIH3}\), an NP-specific IgM was first detected on DPI 1 (2 of 15 positives). This response peaked (100%) on DPIs 7, 8, and 9, with the last positive sample on DPI 21 (Table 2). NP-specific oral fluid IgA was first detected in \(V_{HIH3}\) and \(UV_{HIH3}\) at DPI 1 (2 of 6 pens) and DPI 3 (3 of 14 pens), respectively. Both groups were 100% positive by DPI 7 and remained so for the remainder of the study. All samples from groups \(UV_{HIH3}\) and \(V_{HIH3}\) were positive for NP-specific IgG oral fluid antibody on DPIs 9 through 42.

**Correlation of serum and oral fluid NP isotype-specific antibody responses**

A pen-level evaluation of the association between serum and oral fluid antibody isotype responses was performed using the mean of the serum S/P ratios of pigs within the pen versus the pen-based oral fluid S/P ratios (Figure 3). IgM responses in serum and oral fluid were highly correlated in unvaccinated groups (\(UV_{CTRL}\) and \(UV_{HIH3}\)) \((p < 0.0001, r = 0.810)\). Likewise, serum and oral fluid IgG responses in unvaccinated groups (\(UV_{CTRL}\) and \(UV_{HIH3}\), \(r = 0.839)\) and vaccinated groups (\(V_{CTRL}\) and \(V_{HIH3}\), \(r = 0.856)\) were statistically significant \((p < 0.0001)\). No association between serum and oral fluid antibody responses was found for IgM antibody in vaccinated groups (\(V_{CTRL}\) and \(V_{HIH3}\)) or for IgA antibody in either unvaccinated (\(UV_{CTRL}\) and \(UV_{HIH3}\)) or vaccinated groups (\(V_{CTRL}\) and \(V_{HIH3}\)).
DISCUSSION

The purpose of this study was to describe the kinetics of the NP-specific IgM, IgA, and IgG responses in serum and oral fluid specimens from IAV-vaccinated and non-vaccinated pigs for 42 days following inoculation with IAV H1N1 and H3N2 subtypes. The NP protein, present in all IAV subtypes, is a major component of the ribonucleoprotein complex (El Hefnawi et al., 2011) and a multifunctional, highly-conserved protein with critical roles in IAV replication (Lamb et al., 2001; Portela and Digard, 2002; Shu et al., 1993). Infection with IAV stimulates both cell-mediated and humoral immune responses against NP, including the production of NP-specific cytotoxic T lymphocytes (Braciale et al., 1977; Carragher et al., 2008; Hillaire et al., 2011; Yewdell et al., 2008) and NP-specific IgM, IgA, and IgG antibodies (Carragher et al., 2008; de Boer et al., 1990; Heinen et al., 2000; Heinen et al., 2001; Kim et al., 2006). Subsequent to infection, NP antibody is present in serum (de Boer et al., 1990, Nelson et al., 2001; Sullivan et al., 2009; Ciacci-Zanella et al., 2010) and other sample matrices, e.g., bronchoalveolar lavage (BAL) (Heinen et al., 2000), nasal wash (Heinen et al., 2000), and oral fluid (Panyasing et al., 2012).

Because NP is highly conserved among IAV subtypes, it has been possible to develop antibody assays capable of detecting IAV infections across species. Thus, anti-NP serum antibody was detected in chickens, ducks, ferrets, guinea pigs, horses, humans, mice, seals, and pigs using a double-antibody sandwich blocking ELISA (de Boer et al., 1990). Likewise, anti-NP serum antibody was detected in a variety of marine mammals, including beluga (*Delphinapterus leucas*) and ringed seals (*Phoca hispida*) using a competitive ELISA (Nielson et al., 2001) and in raccoons and mallards using a blocking ELISA (Sullivan et al., 2009). At present, a single commercial NP antibody ELISA (IDEXX Influenza A Ab Test) is available for the detection of NP antibody in chickens, turkeys, ducks, ostriches, geese, pigs, horses, dogs, cats, and zoo animals. IAV IgM has been reported in various specimen types, including serum, bronchoalveolar lavage, and nasal wash samples (Carragher et al., 2008; Heinen et al., 2000; Heinen et al., 2001). IgM is important because it neutralizes IAV early in the course of infection and before other antibody isotypes appear (Ochsenbein et al., 1999). In mice inoculated with
IAV, IgM antibody-secreting cells preceded the appearance of IgG or IgA secreting cells in the lung, spleen, and blood (Jones and Ada., 1986). In contrast, mice unable to produce IgM but capable of producing other antibody isotypes exhibited higher pulmonary IAV titers compared to wild-type mice (Kopf et al., 2002). IgM appears rapidly because it can be produced by B1 cells in the absence of T helper cells via the T-independent pathway, as well as antigen-specific B-cells (B2 cells) via the T-dependent pathway (Baumgarth et al., 1999; Jones et al., 2012). Consistent with prior research, IgM preceded the detection of IgA and IgG in both serum and oral fluids in the present study (Tables 1, 2).

Because of its rapid appearance, it has been suggested that the detection of IgM antibody might be considered indicative of recent IAV infection (Buchner et al., 1977; Jones et al., 2012; Lee et al., 1995). However, the interpretation of IgM results may be complicated by variation in the duration of IAV IgM and the effect of prior immunity to IAV on the IgM response. For example, in humans IAV serum IgM was detectable 42 to 112 days after the onset of symptoms (Buchner et al., 1977). In our study, serum and oral fluid IgM responses were obviated by vaccination. That is, serum IgM was detected in ~98% of unvaccinated pigs on DPI 7, but fewer than 5% of vaccinated pigs. Likewise, oral fluid IgM was detected in 100% of samples collected from pens of unvaccinated pigs on DPI 8, but only 50% of samples from pens of vaccinated pigs. Correspondingly, the serum and oral fluid IgM responses in unvaccinated pigs was highly correlated ($r = 0.81$), but not in vaccinated pigs ($r = -0.04$). Since pigs are commonly vaccinated or infected with IAV early in life in contemporary production systems (USDA, 2008; Yeager et al., 2008), IgM-based assays for serum and oral fluid may have limited utility in the field.

In contrast to low IgA concentration in serum, IAV IgA predominates on mucosal surfaces and functions to prevent the initial attachment of IAV to the epithelial cells of the respiratory tract (Liew et al., 1984). In pigs inoculated with IAV H3N2, the concentration of NP-specific IgA antibodies in bronchoalveolar lavage and nasal wash was estimated at 280 and 570 times, respectively, the concentration in serum at 44 DPI (Heinen et al., 2000). In the current study, significant levels of serum IgA were found only at DPI 14, declining rapidly thereafter. In contrast, the concentration of IgA increased rapidly in oral fluid and remained
elevated through DPI 42. Consistent with these results, the correlation between serum and oral fluid IgA was low \( r \sim 0.30 \). Vaccination interfered with the serum IgA response, but not with the oral fluid IgA response. That is 100% of oral fluid samples from vaccinated and unvaccinated pigs were IgA positive from DPI 7 to 42. Because of the strong IAV IgA response detected in oral fluid and its independence from suppression by vaccination, an oral fluid IgA-based assay might be useful for detecting IAV infections. Future research in this line of inquiry should seek to provide additional information on oral fluid IgA kinetics over time and the response following exposure to homologous or heterologous subtypes.

The inactivated vaccine used in this study induced a detectable serum IgG response in 41% of vaccinated pigs two weeks after the second dose. Following IAV inoculation, vaccinated pigs showed faster and higher serum S/P IgG responses versus unvaccinated pigs. IgG is the major class of serum antibody produced against IAV infection and the primary antibody produced in response to vaccination (Heinen et al., 2001). In contrast to IgM, class-switching is required to increase the affinity of IgG for IAV; which explains why IgG appears later in a primary infection (Graham et al., 1997; Kawabe et al., 1994). IgG present in mucosal secretions is both serum-derived and produced locally (Brokstad et al., 2001; Tew et al., 1985; Wagner et al., 1987), but is not uniformly distributed across mucosal surfaces or among body fluids (Roth, 1992; Spiekermann et al., 2002). Thus, the concentration of IgG1 was found to be 14 times higher in bronchoalveolar lavage fluid than nasal wash (Heinen et al., 2000).

Previous work demonstrated the presence of NP antibody in oral fluids using a blocking ELISA (Panyasing et al., 2012). The current work expanded on these initial observations by describing the kinetics of NP-specific IgM, IgA, and IgG antibodies in oral fluid and serum from IAV-infected pigs. The results of the present study showed a strong correlation \( r > 0.80 \) between serum and oral fluid IgG responses, an observation that justifies further research on the use of oral fluid antibody-based assays as a cost-effective alternative to serum for surveillance of IAV infections in swine populations.
DECLARATION OF CONFLICTING INTERESTS

The author(s) declare that there are no conflicts of interest with respect to their authorship and/or the publication of this manuscript.

FUNDING

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REFERENCES


Table 1. Serum NP isotype-specific antibody ELISAs: Qualitative response by treatment group and day post inoculation (DPI)

<table>
<thead>
<tr>
<th>DPI</th>
<th>IgM&lt;sup&gt;a&lt;/sup&gt; (% positive samples)</th>
<th>IgA&lt;sup&gt;a&lt;/sup&gt; (% positive samples)</th>
<th>IgG&lt;sup&gt;a&lt;/sup&gt; (% positive samples)</th>
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<tr>
<td></td>
<td>UV&lt;sub&gt;CTRL&lt;/sub&gt; (&lt;i&gt;n&lt;/i&gt; = 6)</td>
<td>V&lt;sub&gt;CTRL&lt;/sub&gt; (&lt;i&gt;n&lt;/i&gt; = 6)</td>
<td>UV&lt;sub&gt;H1H3&lt;/sub&gt; (&lt;i&gt;n&lt;/i&gt; = 48)</td>
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<td>42</td>
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<sup>a</sup> ROC analyses of IgM, IgA, and IgG NP ELISA (IDEXX Laboratories, Inc., Westbrook, ME) results established the optimum S/P cut-offs as 0.84 (IgM), 0.75 (IgA) and 0.60 IgG).
Table 2. Oral fluid NP isotype-specific antibody ELISAs: Qualitative response by treatment group and day post inoculation (DPI)

<table>
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<tr>
<th>DPI</th>
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<th>IgA(^a) (% positive samples)</th>
<th>IgG(^a) (% positive samples)</th>
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<tr>
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<td>V(_{CTRL}) (n = 2)</td>
<td>UV(_{HH3}) (n = 16)(^b)</td>
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\(^a\) ROC analyses of IgM, IgA, and IgG ELISA results established the optimum S/P cut-offs as 0.50 (IgM), 0.60 (IgA) and 0.60 (IgG).

\(^b\) Number of samples at DPIs 1 to 5 were 15, 14, 14, 13, and 14, respectively.
Figure 1. Serum influenza virus A nucleoprotein IgM, IgA, and IgG mean antibody responses expressed as sample-to-positive (S/P) ratios over time. Treatment groups included negative control pigs (UV_CTRL), vaccinated control pigs (V_CTRL), and both unvaccinated and vaccinated pigs inoculated with influenza A virus subtype H1N1 or H3N2 (UV_H1H3 and V_H1H3). a First vaccination (Flusure® XP, Pfizer Animal Health, Madison, NJ), b Second vaccination.
Figure 2. Oral fluid influenza virus A nucleoprotein IgM, IgA, and IgG mean antibody responses expressed as sample-to-positive (S/P) ratios over time. Treatment groups included negative control pigs (UVCTRL), vaccinated control pigs (VCTRL), and both unvaccinated and vaccinated pigs inoculated with influenza A virus subtype H1N1 or H3N2 (UVH1H3 and VH1H3).
Figure 3. Pearson's correlation coefficient (r) analysis of pen-level serum and oral fluid influenza virus A nucleoprotein IgM, IgA, and IgG sample-to-positive (S/P) ratios. Unvaccinated groups included negative control pigs (UVCTRL) and unvaccinated inoculated pigs with influenza A virus subtype H1N1 or H3N2 (UVH1H3). Vaccinated groups included vaccinated control pigs (VCTRL) and vaccinated inoculated pigs with influenza A virus subtype H1N1 or H3N2 (VH1H3).
CHAPTER 5. INFLUENZA A VIRUS (IAV) SURVEILLANCE USING PRE-
WEANING PIGLET ORAL FLUIDSAMPLES

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ABSTRACT

The feasibility of conducting influenza A virus (IAV) surveillance using pre-weaning oral fluid samples from litters of piglets was evaluated in four ~12,500 sow, IAV-vaccinated, breeding herds. Oral fluid samples were collected from 600 litters 24 hours prior to weaning. Serum samples from their dams were included for comparison. Litter oral fluid samples were tested for IAV by virus isolation, quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR), RT-PCR subtyping, and sequencing. Commercial nucleoprotein (NP) ELISA kits and NP isotype specific assays (IgM, IgA, and IgG) were used to characterize NP antibody in litter oral fluid and sow serum. All litter oral fluid specimens (n = 600) were negative by virus isolation. Twenty-five oral fluid samples were positive by qRT-PCR, based on screening (Laboratory 1) and confirmatory testing (Laboratory 2). No hemagglutinin (HA) and neuraminidase (NA) gene sequences were obtained, but matrix (M) gene sequences were obtained for all qRT-PCR-positive samples submitted for sequencing (n = 18). Genetic analysis revealed that all M genes sequences were identical (GenBank accession no. KF487544) and belonged to the triple reassortant influenza A virus M gene (TRIG M) previously identified in swine. The proportion of IgM- and IgA-positive samples was significantly higher in sow serum and litter oral fluid samples, respectively (p < 0.01). Consistent with the extensive use of IAV vaccine, no difference was detected in the proportion of IgG- and blocking ELISA-positive sow serum and litter oral fluids. This study supported the use of oral fluid sampling as a means to conduct IAV surveillance in pig populations and demonstrated the inapparent circulation of IAV in piglets. Future work on IAV oral fluid diagnostics should focus on improved procedures for virus isolation,
subtyping, and sequencing of HA and NA genes. The role of antibody in IAV surveillance remains to be elucidated, but longitudinal assessment of specific antibody has the potential to provide information regarding patterns of infection, vaccination status, and herd immunity.

INTRODUCTION

Influenza A virus (IAV) is infectious for a wide variety of vertebrate hosts and is important as a cause of acute respiratory disease in humans and domestic animal species (Vincent et al., 2008). Historically, IAV has been associated with major epidemics in humans, horses, poultry, and swine (Morens and Taubenberger, 2010; Shope, 1958). IAV is an enveloped RNA virus composed of 8 negative-sense, single-stranded genomic segments that undergo rapid genetic evolution via point mutation and genetic reassortment (Brown, 2000). Genetically diverse, IAV subtype classification is based on two external surface proteins, hemagglutinin (HA) and neuraminidase (NA). HA facilitates the attachment of the virus to epithelial cells in the respiratory tract via sialic acid (SA) molecules bound to galactose (Gal). Among its functions, NA cleaves SA from cells to release progeny virus. To date, 17 HAs and 10 NAs have been identified (Tong et al., 2012; Zhu et al., 2012). HAs from avian IAVs preferentially bind to SAα2,3-Gal receptors while HAs from mammalian IAVs have an affinity for SAα2,6-Gal receptors (Gagneux et al., 2003; Matrosovich et al., 1999). This is important because differences in the distribution of SAα2,3-Gal and SAα2,6-Gal receptors in the respiratory tract reflect host species susceptibility to IAVs. In quail (Coturnix coturnix), pheasants (ring-necked pheasants, Phasianus colchicus), chickens (Gallus gallus), Pekin ducks (Anas platyrhynchos domestica), both SAα2,3-Gal and SAα2,6-Gal receptors are present throughout the respiratory system (Yu et al., 2011). In contrast, SAα2,6-Gal receptors predominate in the upper respiratory tracts of humans and pigs and SAα2,3-Gal receptors in the lower (Nelli et al., 2010; Shinya et al., 2006).

Influenza A virus in swine is a public health issue because of concerns that reassortants originating in pigs could spill over to susceptible human populations. However, IAV in swine is also an animal welfare issue because of its impact on pig health and productivity. Control of IAV in swine populations is complicated by the fact that the virus is endemic in
contemporary herds and may circulate in any age group, including suckling pigs (Corzo et al., 2012). Commonly, more than one subtype circulates concurrently in a population (Corzo et al., 2013). Ultimately, the control of IAV in swine will rely on effective interventions based on a sound understanding of the ecology of IAV in contemporary swine production systems. This fundamental understanding can only be achieved by collecting longitudinal data in commercial swine herds, i.e., surveillance. Traditionally, IAV surveillance has been based on collecting and testing individual pig nasal swab and/or serum samples, but the labor and cost of this approach makes it unacceptable for routine use. A possible solution is surveillance based on pen-based oral fluid specimens. Previous research showed that both IAV and anti-IAV antibody can be detected in oral fluid specimens using quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) and enzyme-linked immunosorbent assay (ELISA), respectively (Detmer et al., 2011; Panyasing et al., 2012; Ramirez et al., 2012; Romagosa et al. 2012). The purpose of the current study was to evaluate the feasibility of IAV surveillance in breeding herds using pre-weaning oral fluid samples from litters of piglets.

**MATERIALS AND METHODS**

**Experimental Design**

In four IAV-vaccinated commercial swine herds, oral fluid samples were collected from 600 litters 24 h prior to weaning and serum samples from their dams ≤ 48 h later. Thereafter, samples were completely randomized within specimen type and tested. Oral fluid samples were assayed for IAV by virus isolation and qRT-PCR, followed by subtyping and sequencing on qRT-PCR-positive samples. Both sow serum and litter oral fluid specimens were tested for IAV nucleoprotein (NP) antibody using a commercial NP blocking ELISA (IDEXX influenza A Ab Test, IDEXX Laboratories, Inc., Westbrook, ME) and NP isotype-specific indirect ELISAs (IgM, IgA, and IgG). Descriptive and comparative statistical analyses were used to evaluate and compare quantitative and qualitative results in serum and oral fluid samples, as well as significant associations with animal and herd factors.
Animals and animal care
The study was conducted under the approval of the Iowa State University Institutional Animal Care and Use Committee (#8-11-7202-S). The study was performed in four ~12,500 sow breeding herds located in Oklahoma, USA. Farm A was located ~0.8 km (0.5 miles) from Farm B and Farm C the same distance from Farm D. Farms A/B were ~9.7 km (6 miles) from farms C/D. Gilts sources, animals housing, feeding, handling, and veterinary care were under the supervision of Seaboard L.L.C. Health Assurance and Welfare personnel. All four herds were considered to be endemically infected with IAV on the basis of their diagnostic history. Replacement gilts were routinely vaccinated with autogenous IAV vaccine (Newport Laboratories, Worthington, MN) at approximately 9, 12, and 24 weeks of age and again one week post farrowing.

Sample collection
Six hundred pairs of sow serum and pre-weaning litter oral fluid samples were collected over a four month period by farm personnel (Table 1). Piglets averaged 17 days of age (range: 15 to 19 days) at the time of collection. Oral fluid samples were collected from litters one day prior to weaning by suspending unbleached cotton rope (1.25 cm, 0.5 in) in the area of the heat mat and within access of the piglets. When the material was saturated (15 min to 2 h), oral fluid was extracted by manually squeezing the rope while inside a plastic bag. Thereafter, the sample was transferred into a tube, held at 4°C, and shipped to the laboratory on the following day. At the laboratory, samples were aliquoted into 5 ml cryogenic vials and stored at -80°C. Within 48 h of litter oral fluid collection, sows were bled using a single-use blood collection system (Corvac®, Tyco Healthcare Group LP, Mansfield, MA) and samples were shipped overnight to the laboratory. At the laboratory, samples were centrifuged at 1,000 x g for 10 min, after which the serum was aliquoted into 5 ml cryogenic vials and stored at -80°C. When all samples had been collected, they were completely randomized and tested.

Virus isolation (oral fluid)
Confluent monolayers of MDCK cells were prepared in 48-well plates (Costar, Corning, Corning, NY). Cell culture media was removed and monolayers were washed 3 times with
IAV wash solution composed of minimal essential medium with Earle’s salts (MEM; Sigma-Aldrich, St. Louis, MO), 3x antibiotic-antimycotic solution [penicillin (300 IU/ml; Sigma-Aldrich), streptomycin (300 μg/ml; Sigma-Aldrich), gentamicin (150 μg/ml; Sigma-Aldrich) and amphotericin B (0.75 μg/ml; Gibco, Grand Island, NY)], and TPCK-treated trypsin (2 μg/ml; Sigma-Aldrich). Prior to inoculation onto MDCK cells, 0.35 μl of antibiotic-antimycotic solution was added to each 1 ml of oral fluid, after which samples were held at room temperature for 1 h. Each oral fluid sample was divided among 3 wells, i.e., ~0.4 ml/well, and then incubated at 37°C with 5% CO2 for 2 h, after which the inoculum was removed. Cell monolayers were rinsed 3 times with the IAV wash solution, and then 0.4 ml IAV post-inoculation media composed of MEM with Earle's salts, 3x antibiotic-antimycotic solution, and TPCK-treated trypsin (1.5 μg/ml) was added and cell cultures were incubated for up to 5 days. Cell cultures were evaluated for the appearance of cytopathic effect (CPE) daily. If CPE was present, cell culture fluid was tested for HA activity and HA-positive cell culture fluids were tested for IAV by qRT-PCR. Cells with no CPE were subjected to 2 freeze-thaw cycles (-80°C and 37°C) and tested for HA activity. Samples negative for CPE and/or HA were subjected to a second cell culture passage by pooling the fluid from all 3 wells and then re-inoculating fresh confluent MDCK cells in 3 wells. Samples were considered negative if CPE and HA were negative after the second passage on cell culture. Contaminated cell culture fluids were considered “not determined”.

**Influenza A virus oral fluid qRT-PCR (Laboratory 1)**

RNA extraction was performed on oral fluid specimens using the MagMAX™ Viral RNA Isolation Kit (Life Technologies, Carlsbad, CA) and a Kingfisher 96 instrument (Thermo Scientific, Waltham, MA) using the high volume modified lysis (HVML) procedure. The lysis/binding solution for the HVML protocol was prepared using 45 ml lysis/binding solution with 200 μl carrier RNA without the addition of isopropanol. For the lysis step, 300 μl of sample was added to 450 μl of modified lysis/binding solution. Xeno™ internal control RNA was added to the lysis-binding solution at 4,000 copies per reaction prior to extraction to monitor PCR amplification and detect inhibition. The sample, lysis(binding solution, and internal control RNA were vortexed for 3 min and centrifuged at 2,500 x g for 6 min. A volume of 600 μl of lysate was added to 350 μl isopropanol with 20 μl magnetic bead mix
prior to extraction and elution into 90 μl buffer. The HVML used 300 and 450 μl of wash solutions I and II, respectively. The HVML extraction was conducted using the Kingfisher program AM1836_DW_HV_v3.

Influenza A virus qRT-PCR was performed on nucleic acid extracts according to the manufacturer's instructions using PCR reagents with multiple primers and probes targeting different genomic regions (MagMAX™ Gold SIV Detection Kit, Life Technologies). One positive extraction control, one positive amplification control, one negative extraction control, and a negative amplification control were included with each extraction and/or PCR run. Each oral fluid reaction included 12.5 μl of 2X multiplex RT-PCR buffer, 1.0 μl of 25X SIV primer probe mix, 2.5 μl of 10X multiplex RT-PCR enzyme mix, and 1.0 μl of nuclease-free water. A final volume of 25 μl, consisting of 17 μl master mix and 8 μl of RNA extract, was placed in each well of a 96-well fast PCR plate (Life Technologies). qRT-PCR was performed using an AB 7500 fast thermocycler: 1 cycle at 48°C for 10 min, 1 cycle at 95°C for 10 min, and 40 cycles of 95°C for 15 sec, 60°C for 45 sec. Amplification curves were analyzed with commercial thermal cycler system software. The cycle threshold was set at 0.2 and the “auto baseline” was used to determine fluorescence baselines. Samples with Ct values < 38 were considered positive. Internal control Xeno™ RNA Ct values were set at 10% of maximum.

Influenza A virus oral fluid qRT-PCR (Laboratory 2)

Oral fluid samples identified as IAV qRT-PCR positive (n = 22) at Laboratory 1 were submitted with qRT-PCR negative oral fluid samples (n = 44) to Laboratory 2 for confirmatory testing (Tetracore®, Inc., Rockville, MD).

Prior to RNA extraction, 180 μl of sample was centrifuged (14,000 x g for 30 sec) and then 140 μl of the supernatant was manually lysed in a biosafety cabinet. Nucleic acids were extracted and purified from the lysate according to the manufacturer’s recommendations using the QIAGEN® QIAamp® Viral Mini QIAcube® kit (Catalog #52926, Qiagen, Inc., Valencia, CA) on the QIAGEN® QIAcube® processor (Qiagen, Inc.). The inhibition control (IC) was used as an extraction and PCR inhibition control for each sample.
The qRT-PCR procedure was performed using commercial reagents (Universal Influenza A Matrix MPX 2.0, Tetracore®, Inc.) and the dry master mix was prepared according to the manufacturer’s recommendations. The reactions were run (Applied Biosystems® 7500 Fast Real-Time PCR System, Applied Biosystems®, Foster City, CA): 50°C for 30 min (reverse transcription), then 95°C for 2 min (RT inactivation/initial denaturation), followed by 40 cycles of 95°C for 15 sec, 52°C for 15 sec, and 60°C for 33 sec (amplification). The thermocycler was run in “standard” mode and fluorescence data was collected during the 60°C step in the FAM™ and CY5 channels. A sample was considered positive for the IAV matrix target if it yielded a Ct of < 37.

Influenza A virus subtyping (Laboratory 1)
Hemagglutinin (HA) and neuraminidase (NA) subtyping was performed on qRT-PCR-positive IAV nucleic acid extracts using Swine Influenza Virus Subtyping RNA Reagents (Life Technologies, Carlsbad, CA). Separate RT-PCR reactions were used to detect the presence of H1 or H3 HA or N1 or N2 NA, respectively. Each oral fluid reaction included 12.5 μl of 2X multiplex RT-PCR buffer, 1.0 μl of 25X H1H3 or N1N2 Primer Probe Mix, 2.5 μl of 10X multiplex RT-PCR enzyme mix, and 1.0 μl of nuclease-free water. Each subtyping plate included the same positive and negative controls used in the SIV general RT-PCR reaction. The IAV general RT-PCR cycling conditions were used for RT-PCR subtyping and amplification curves were analyzed with commercial thermal cycler system software using the same cycle threshold and “auto baseline” determinants as the IAV general RT-PCR. Samples with Ct values < 38 were considered positive.

Influenza A virus sequencing (HA, NA and M gene)
Whole genome HA, NA, and matrix (M) genes were sequenced using conventional methods. Viral RNA was extracted using the Ambion MagMAX™-96 AI/ND (Life Technologies) and a Kingfisher 96 instrument (Thermo Scientific). Specifically, 50 μl of sample, 100 μl of viral lysis/binding solution with carrier RNA, and 20 μl of bead solution were used with 100 μl of the wash solution supplied with the kit. The final extracted sample elution volume was 50 μl. The extractions were performed using the Kingfisher program AM_1835_DW_NVSL. RT-PCR was conducted for each gene segment using the primers described in Table 2 and the
FideliTaq™ RT-PCR Master Mix (2X) kit (Affymetrix, Cleveland, OH). The sequencing RT-PCR setup reaction used 200 nM of each primer with 25 μl 2X RT-PCR master mix and 9 μl or 13 μl nuclease-free water for the HA or NA/M genes, respectively. The final volume of 50 μl consisted of 38 μl or 42 μl master mix and 12 μl or 8 μl of RNA extract for the HA or NA/M genes, respectively. One positive extraction control (H1 or H3), one negative extraction control, and one negative amplification control were included with the reaction. RT-PCR was performed using an ABI 2720 thermal cycler: 1 cycle at 48°C for 30 min, 1 cycle at 95°C for 3 min, 50 cycles of 94°C for 30 sec, 48°C for 30 sec, and 68°C for 120 sec. The final elongation step was 68°C for 5 min. Detection of the RT-PCR product, HA at 1800 base pairs (bp), the NA at 1500 bp and M at 1100 bp, was performed on a QIAxcel® (Qiagen, Inc.) capillary electrophoresis system using a DNA screening cartridge and the AM420 method and purified with ExoSAP-IT PCR cleanup reagent (Affymetrix) following the manufacturer’s recommendations. Samples were submitted to the Iowa State University DNA facility (Ames, IA) for sequencing. Lasergene® software (DNASTar, Madison, WI) was used to compile sequences.

**Serum NP blocking ELISA**

Sow serum samples were tested for NP antibodies using a commercial blocking ELISA performed as recommended by the manufacturer (IDEXX Influenza A Ab Test, IDEXX Laboratories, Inc.). Reactions were measured as optical density (OD) at a wavelength of 650 nm using an ELISA plate reader (BioTek® Instruments Inc., Winooski, VT), operated with commercial software (GEN5™, BioTek® Instruments Inc.). Sample-to-negative (S/N) ratios were calculated as described by the manufacturer, with S/N ratios \( \leq 0.60 \) considered antibody positive (Goodell et al., 2013a).

**Serum NP isotype-specific indirect ELISAs**

Sow serum samples were assayed for anti-NP IgM, IgA, and IgG using indirect ELISAs. Plates and reagents (sample diluent, substrate, stop solution, and wash solution) were from a commercial IAV blocking ELISA (IDEXX Influenza A Ab Test, IDEXX Laboratories, Inc.) and conjugate diluent from a commercial indirect ELISA (IDEXX PRRS X3 Ab Test,
IDEXX Laboratories, Inc.). Detection of isotype-specific NP antibody utilized horseradish peroxidase (HRP) conjugated goat anti-pig IgM (A100-100P), IgA (A100-102P), and IgG (A100-104P) antibody (Bethyl Laboratories Inc., Montgomery, TX) diluted in conjugate diluent. Duplicate in-house negative and positive serum controls (IgM, IgA, and IgG) were used to validate plate performance and to calculate sample-to-positive (S/P) ratios.

To perform the assay, serum samples were diluted 1:50 (IgM), 1:10 (IgA), or 1:50 (IgG), then plates were loaded (100 µl) with samples, negative and positive controls (IgM, IgA, or IgG). After 2 h incubation at 37°C, plates were washed 3 times, then 100 µl of diluted HRP-conjugated goat anti-pig IgM (1:8,000) or IgA (1:2000) or IgG (1:10,000) was added to each well and the plates incubated at 37 °C for 1 h. Plates were then washed 3 times, after which 100 µl of 3, 3’, 5, 5’- tetramethyl benzidine (TMB) substrate was added. After 15 min incubation at room temperature, 100 µl of stop solution was added and plates read at a wavelength of 650 nm. S/P ratios were calculated for each sample as:

\[
S/P \text{ ratio} = \frac{\text{sample OD} - \text{negative control mean OD}}{\text{positive control mean OD} - \text{negative control mean OD}}
\]

**Oral fluid NP blocking ELISA**

Oral fluid samples were assayed for IAV antibody using a commercial blocking ELISA (IDEXX Influenza A Ab Test, DEXX Laboratories, Inc.) performed as described elsewhere (Panyasing et al., 2012). Briefly, each plate was loaded with undiluted oral fluid samples (200 µl), kit negative and positive controls, and in-house oral fluid controls (low, medium, high) and incubated for 16 h at 22 °C. Thereafter, the assay was performed and S/N ratios calculated as described by the manufacturer.

**Oral fluid NP isotype-specific indirect ELISAs**

The oral fluid NP isotype-specific indirect ELISAs are described elsewhere (Panyasing et al., 2013). Briefly, oral fluid samples were assayed on ELISA plates manually coated with one µg/well of commercially-produced NP antigen (Cat no. IMR-274, Imgenex© Corporation,
San Diego, CA). The reagents used in the ELISAs were identical to the serum NP-isotype specific assays, with the exception of IgM, IgA, and IgG conjugate concentrations. To perform the assays, plates were loaded with undiluted oral fluid specimens (200 µl) and prediluted negative (100 µl) and positive (100 µl) in-house plate controls (in duplicate) and incubated at 4°C for 16 h. After incubation, plates were washed 3 times. To detect IgM, IgA, and IgG, 100 µl of HRP-conjugated goat anti-pig IgM (1:2,000), IgA (1:2000) or IgG (1:1,500) was added and the plates incubated at 37°C for 1 h. Plates were then washed 3 times, TMB substrate (100 µl) added, and the plates incubated at room temperature for 15 min. Stop solution (100 µl) was then added and the plates read immediately thereafter. Reactions were measured at a wavelength of 650 nm and S/P ratios calculated.

**Statistical analysis**

Oral fluid qRT-PCR qualitative responses were analyzed for significant differences among herds and sampling time points (Fisher’s Exact test) and significant associations with sow parity and litter size (Wilcoxon-Mann-Whitney test) using commercial software (SAS® 9.2, SAS® Institute Inc., Cary, NC). The M gene nucleotide sequences were compared with a variety of classical and recent North American swine-derived IAV full-length M gene sequences available in the GenBank database. A phylogenetic tree was generated by the distance-based, neighbor-joining method using MEGA5.2 software (Tamura et al., 2011). Serum and oral fluid S/P and S/N antibody responses were analyzed for significant associations with sow parity, litter size, herd, sampling time point, qRT-PCR response, and their interactions by analysis of variance (ANOVA). The relationship between IgM, IgA, and IgG S/P ratios in serum versus oral fluid samples was evaluated by correlation analysis (Pearson’s correlation coefficient).

**RESULTS**

**Detection of Influenza A virus in oral fluid**

All oral fluid specimens (n = 600) were negative by virus isolation. IAV qRT-PCR testing in Laboratory 1 identified 22 (3.7 %) positive oral fluid samples (Table 3). Ten of the 22 positive samples were successfully subtyped, with most shown to be mixed infections (H1,
To confirm the qRT-PCR results, the 22 positive samples plus 44 randomly-selected qRT-PCR-negative samples were submitted to Laboratory 2. Testing at Laboratory 2 identified 18 qRT-PCR positive samples, including 3 that had previously tested negative at Laboratory 1. Among the cumulative total of 25 qRT-PCR positive samples reported by Laboratory 1 and/or Laboratory 2, 18 were available for sequencing, i.e., 7 samples had been depleted. None of the attempts to sequence HA and NA genes was successful, but M gene sequences were obtained for all 18 samples. Genetic analysis revealed that the 18 M gene nucleotide sequences were 100% identical to each other (GenBank Accession number KF487544) and to the M gene of a previous GenBank submission (JX444793/A/swine/Ohio/A01203624/2012(H3N2) (Figure 1).

As shown in Table 1, the cumulative proportion of IAV qRT-PCR-positive oral fluid samples by farm (high to low) was: Farm B (15/145, 10.3%), Farm D (7/150, 4.7%), Farm A (2/153, 1.3%), Farm C (1/152, 0.7%). Pairwise comparisons showed that the proportion of qRT-PCR positives in Farm B differed significantly from Farms A and C (Fisher's Exact Test, $p < 0.001$), but not from Farm D ($p = 0.07$). In Farm B, 14 of the 15 total qRT-PCR positive oral fluid samples were recovered at one sampling point, i.e., calendar week 28. Similarly in Farm D, 5 of the 7 total qRT-PCR positive oral fluid samples were detected in the samples collected at week 30. No association was detected between IAV qRT-PCR oral fluid status and sow parity or the number of pigs weaned (Wilcoxon Mann-Whitney test, $p > 0.05$).

**IAV NP antibody in sow serum and litter oral fluid samples**

Qualitative results of IAV NP antibody testing of sow serum and litter oral fluid are shown in Table 4. The proportion of IgM, IgA, IgG, or blocking ELISA-positive sow serum or litter oral fluid samples did not differ among herds or within farms by calendar week. A comparison of sow serum and litter oral fluids qualitative results found no difference in the proportion of IgG or blocking ELISA positives, but the proportion of IgM and IgA positives was higher in sow serum and litter oral fluid, respectively (Fisher’s Exact Test, $p < 0.01$). No association was detected between IAV qRT-PCR oral fluid results and IgM, IgA, IgG, or blocking ELISA qualitative results in sow serum or litter oral fluid samples (Fisher’s Exact Test, $p > 0.20$).
Sow serum and litter oral fluid IgM, IgA, IgG, and blocking ELISA quantitative responses (S/P and S/N ratios) are shown in Figure 2. No significant association was detected between sow serum or litter oral fluid antibody responses and farm, sow parity, number of pig weaned, calendar week, or IAV qRT-PCR oral fluid results. Evaluation of the association between litter oral fluid and sow serum antibody responses showed a significant association between IgG S/P ratios ($p = 0.01, r = 0.10$), but not between IgM, IgA, or blocking ELISA responses.

**DISCUSSION**

Influenza A virus surveillance in susceptible species is necessary to monitor viral evolution and support the development of improved diagnostic tests and more efficacious vaccines (Vincent et al., 2013). More narrowly, research on the ecology of IAV in swine populations is motivated by concerns that reassortants originating in pigs could prove to be virulent in humans and by the need to ameliorate the negative effects of IAV on pig welfare and health. Historically, the collection of longitudinal infectious disease data in commercial herds has been constrained by the expense and inconvenience of collecting and testing specimens from individual animals. As an alternative, we evaluated the feasibility of conducting IAV surveillance using oral fluid samples collected from litters of piglets prior to weaning. This age group was selected under the premise that surveillance data from this group would allow producers to identify and respond to health issues in the breeding herd and anticipate post-weanling disease issues in growing pig populations. Serum samples collected from each litters' dam provided for comparisons with litter oral fluid testing results. Oral fluid and serum samples were collected by farm personnel and then sent to the laboratory for testing. This approach avoided the biosecurity risks invariably associated with sending outside personnel into a herd to collect samples.

The results of the study showed that IAV could be detected by qRT-PCR, subtyped, and sequenced using oral fluid specimens collected from neonatal pigs. All attempts at virus isolation from oral fluids were negative, but virus subtyping revealed that infections were commonly a mix of subtypes H1, H3, N1, and N2. Cumulatively, the results showed a pattern of intermittent subclinical IAV infections in litters from IAV-vaccinated dams, with
infrequent episodes involving larger numbers of litters. These results are in accord with the view that IAV circulates throughout the year in contemporary swine populations (Van Reeth et al., 2012). The absence of clinical losses was consistent with reports that maternal immunity can moderate fever, reduce clinical signs, and prolong virus shedding, but not prevent infection (Allerson et al., 2013; Kittikoon et al., 2006; Loeffen et al., 2003). These results were compatible with previous reports. Evaluating the detection of IAV in oral fluids by qRT-PCR as a function of within-pen prevalence, Romagosa et al. (2012) estimated the probability of detecting one acutely-infected pig in a pen of 11 at 69% and 2 infected pigs at 99%. Detmer et al. (2011) reported successful subtyping, HA gene sequencing, and IAV isolation from oral fluid field samples, albeit virus isolation is generally more successful using nasal swabs (Goodell et al., 2013b).

Although the study was not designed to compare assay reproducibility, serial testing of a subset of samples showed that false negative results occurred in both laboratories performing qRT-PCR testing. That is, IAV M gene sequencing was successful on all qRT-PCR-positive samples submitted for sequencing (n = 18, Table 3), even if the sample tested negative in one of the two laboratories. HA and NA gene sequences were not obtained, but analysis of M gene sequences (n = 18) showed 100% nucleotide identity to each other and a previous GenBank submission in the TRIG M cluster (JX444793/A/swine/Ohio/A01203624/2012(H3N2)). The length and genetic diversity of the HA (~1,700 nucleotides) and NA (~1,400 nucleotides) genes may account for the difficulty in sequencing these genes, as opposed to the highly conserved and shorter M gene (~1,000 nucleotides) (Lamb and Krung, 2001). This problem may be resolved in the future as assays are improved.

Previous research described the ontogeny of IAV NP antibody responses over time in oral fluid (Panyasing et al., 2012), serum, bronchoalveolar and nasal lavage fluid (Heinen et al., 2000) from pigs under experimental conditions. In the present study, nearly all serum and oral fluid samples were positive for NP IgG antibody due to the extensive use of IAV vaccine and the concomitant circulation of wild-type IAV in these populations. Although IgM and/or IgA positive maternal serum and piglet oral fluid samples were identified, no association was detected between IAV qRT-PCR results and NP antibody profiles (IgM, IgA, IgG, and
blocking ELISAs). In part, the ability to detect patterns of antibody response to infection was compromised by the experimental design, i.e., samples were collected at a single point in time from each litter or sow.

Overall, the present study supports the use of oral fluid sampling as a means to conduct IAV surveillance in pig populations. In particular, oral fluid offers the potential to conduct surveillance with fewer samples than required for individual pig testing. Future work on IAV oral fluid diagnostics should focus on improved procedures for virus isolation, subtyping, and sequencing of HA and NA genes. The role of antibody in IAV surveillance remains to be determined, but longitudinal assessment of specific antibody could provide information regarding patterns of infection, vaccination status, and herd immunity.

CONFLICT OF INTEREST

The author(s) declare no conflicts of interest with respect to their authorship and/or the publication of this manuscript. Dr. C. Goodell is currently employed by IDEXX Laboratories, Inc., Westbrook, ME. R. Rauh is employed at Tetracore®, Inc., Rockville, MD.

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REFERENCES


Table 1. Count of matched sow serum and litter oral fluid (OF) samples and influenza A virus (IAV) qRT-PCR-positive OF samples

<table>
<thead>
<tr>
<th>Samples by farm&lt;sup&gt;a&lt;/sup&gt;</th>
<th>May</th>
<th>June</th>
<th>July</th>
<th>August</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Farm A - samples (serum and OF)</td>
<td>11</td>
<td>•</td>
<td>•</td>
<td>33</td>
<td>33</td>
</tr>
<tr>
<td>IAV qRT-PCR-positive OF samples</td>
<td>0</td>
<td>•</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Farm B - samples (serum and OF)</td>
<td>18</td>
<td>5</td>
<td>17</td>
<td>•</td>
<td>15</td>
</tr>
<tr>
<td>IAV qRT-PCR-positive OF samples</td>
<td>1</td>
<td>0</td>
<td>•</td>
<td>•</td>
<td>14</td>
</tr>
<tr>
<td>Farm C - samples (serum and OF)</td>
<td>•</td>
<td>•</td>
<td>•</td>
<td>•</td>
<td>•</td>
</tr>
<tr>
<td>IAV qRT-PCR-positive OF samples</td>
<td>•</td>
<td>•</td>
<td>•</td>
<td>•</td>
<td>•</td>
</tr>
<tr>
<td>Farm D - samples (serum and OF)</td>
<td>•</td>
<td>•</td>
<td>•</td>
<td>•</td>
<td>•</td>
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<tr>
<td>IAV qRT-PCR-positive OF samples</td>
<td>•</td>
<td>•</td>
<td>•</td>
<td>•</td>
<td>•</td>
</tr>
<tr>
<td>Total samples (serum and OF)</td>
<td>29</td>
<td>5</td>
<td>17</td>
<td>0</td>
<td>33</td>
</tr>
<tr>
<td>IAV qRT-PCR-positive OF samples</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<sup>a</sup> All gilts were vaccinated with autogenous influenza A virus vaccine at approximately 9, 12, and 24 weeks of age and then 1 week post farrowing.
Table 2. Primer sets used for RT-PCR amplification of hemagglutinin (HA), neuraminidase (NA) and matrix (M) genes of influenza A viruses

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer</th>
<th>Primer Sequence 5'–3'</th>
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<tbody>
<tr>
<td>H1</td>
<td>H1-F</td>
<td>AAGCAAAAGCAGGGGAAATAA</td>
</tr>
<tr>
<td></td>
<td>HR</td>
<td>AGTAGAAACAAAGGGTTTTTT</td>
</tr>
<tr>
<td>H3</td>
<td>H3-F</td>
<td>AGCAAAAGCAGGGGATAATTCT</td>
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<tr>
<td></td>
<td>HR</td>
<td>AGTAGAAACAAAGGGTTTTTT</td>
</tr>
<tr>
<td>NA</td>
<td>NA-1F</td>
<td>TAT TGG TCT CAG GGA GCA AAA GCA GGA GT</td>
</tr>
<tr>
<td></td>
<td>NA-1413R</td>
<td>ATA TGG TCT CGT ATT AGT AGA AAC AAG GAG TTT TTT</td>
</tr>
<tr>
<td>M</td>
<td>M-1F</td>
<td>TAT TCG TCT CAG GGA GCA AAA GCA GGT AG</td>
</tr>
<tr>
<td>M</td>
<td>M-1027R</td>
<td>ATA TCG TCT CGT ATT AGT AGA AAC AAG GTA GTT TTT</td>
</tr>
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</table>
Table 3. Summary of test results on influenza A virus qRT-PCR-positive litter oral fluid samples and matched sow serum

<table>
<thead>
<tr>
<th>Farm</th>
<th>Sow parity</th>
<th>Pigs weaned</th>
<th>IgM (S/P)</th>
<th>IgA (S/P)</th>
<th>IgG (S/P)</th>
<th>Lab1&lt;sup&gt;a&lt;/sup&gt; Ct qRT-PCR</th>
<th>Lab2&lt;sup&gt;b&lt;/sup&gt; Ct qRT-PCR</th>
<th>Virus typing</th>
<th>M gene sequencing</th>
<th>IgM (S/P)</th>
<th>IgA (S/P)</th>
<th>IgG (S/P)</th>
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<tbody>
<tr>
<td>A</td>
<td>3</td>
<td>11</td>
<td>0.63</td>
<td>0.18</td>
<td>2.92</td>
<td>&gt;38.0</td>
<td>36.62</td>
<td>ND</td>
<td>Y</td>
<td>0.40</td>
<td>0.01</td>
<td>2.75</td>
</tr>
<tr>
<td>A</td>
<td>2</td>
<td>12</td>
<td>0.17</td>
<td>0.58</td>
<td>2.51</td>
<td>37.70</td>
<td>&gt;37.0</td>
<td>Untypable</td>
<td>Y</td>
<td>0.02</td>
<td>0.12</td>
<td>2.87</td>
</tr>
<tr>
<td>B</td>
<td>4</td>
<td>10</td>
<td>0.52</td>
<td>0.75</td>
<td>2.62</td>
<td>30.70</td>
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<sup>a</sup> Cts < 38 were positive in Laboratory 1.  
<sup>b</sup> Cts < 37 were positive in Laboratory 2.  
<sup>c</sup> Quantity not sufficient (QNS) for testing.
Table 4. **Influenza A virus nucleoprotein (NP) antibody ELISA (IgM, IgA, IgG, blocking) qualitative testing results**

<table>
<thead>
<tr>
<th>Farm&lt;sup&gt;a&lt;/sup&gt;</th>
<th>n</th>
<th>IgM&lt;sup&gt;b&lt;/sup&gt;</th>
<th>IgA&lt;sup&gt;b&lt;/sup&gt;</th>
<th>IgG&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Blocking&lt;sup&gt;c&lt;/sup&gt;</th>
<th>IgM&lt;sup&gt;d&lt;/sup&gt;</th>
<th>IgA&lt;sup&gt;d&lt;/sup&gt;</th>
<th>IgG&lt;sup&gt;d&lt;/sup&gt;</th>
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<td>145</td>
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<tr>
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<td>568</td>
<td>6</td>
<td>39</td>
<td>599</td>
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<sup>a</sup> All gilts were vaccinated with autogenous influenza A virus vaccine at approximately 9, 12, and 24 weeks of age and then 1 week post farrowing.

<sup>b</sup> Indirect ELISA S/P ratios $\geq 0.84$ (IgM), $\geq 0.75$ (IgA) or $\geq 0.60$ (IgG) were considered antibody positive (Panyasing et al., 2013).

<sup>c</sup> Blocking ELISA S/N ratios $\leq 0.60$ were considered antibody positive (Goodell et al., 2013a).

<sup>d</sup> Indirect ELISA S/P ratios $\geq 0.50$ (IgM), $\geq 0.60$ (IgA) or $\geq 0.60$ (IgG) were considered antibody positive (Panyasing et al., 2013).

<sup>e</sup> Blocking ELISA S/N ratios $\leq 0.60$ were considered antibody positive (Panyasing et al., 2012).
Figure 1. Phylogenetic tree based on an analysis of the matrix (M) genes of influenza A viruses detected in North American swine available through GenBank. The viruses detected in this study are indicated with a solid diamond ♦. The tree was constructed using the distance-based, neighbor-joining method (MEGA5.2; Tamura et al., 2011). The reliability of the tree was assessed by a bootstrap analysis with 1000 replications. Scale bars indicate nucleotide substitutions per site.
Figure 2. Influenza A virus nucleoprotein (NP) antibody ELISA (IgM, IgA, IgG, blocking) quantitative testing results.
CHAPTER 6. GENERAL CONCLUSIONS

Influenza A virus (IAV) research in swine began with Shope's (1931) work with "infectious material" in 1930. This line of research continues in full force today because IAV remains endemic in pig populations, causing significant economic losses to pork producers and fueling on-going public health concerns. Despite 80-plus years of research, we lack the knowledge to achieve effective control of IAV in swine. We suggest that one of the missing elements is an understanding of IAV at the population level and that this lack can only be provided by surveillance.

Viable surveillance systems should be designed based on the criteria described by Klaucke et al. (1998): simple (easy to operate), flexible (adaptable to changing conditions and needs), acceptable (individuals and organizations choose to participate), timely (data enter the system quickly), and accurate. Animal disease surveillance programs have historically met few of these criteria. To the contrary, surveillance has historically been labor-intensive, costly, and slow -- attributes that have generally precluded it from achieving the utility necessary for it to fulfill its function (Pappaioanou and Gramer, 2010).

The use of oral fluid sampling can expedite IAV surveillance in swine populations because it is welfare-friendly, worker-friendly, cost-effective, and diagnostically efficient. For example, IAV qRT-PCR testing of pen-based oral fluids showed increased analytical sensitivity when compared to individual animal samples (Romagosa et al. 2012). Evaluating the detection of IAV in oral fluids by qRT-PCR as a function of within-pen prevalence, Romagosa et al. (2012) estimated the probability of detecting one acutely-infected pig in a pen of 11 at 69% and 2 infected pigs at 99%. The work reported in this dissertation showed that this approach is also amenable to the genetic characterization of IAV isolates using molecular technology, e.g., RT-PCR subtyping and sequencing (Chapter 5).

Antibody-based assays have historically played a large role in surveillance. In the case of IAV, antibody detected in oral fluid had a marked advantage over virus detection by isolation or RT-PCR in terms of the longevity of detection. That is, IAV is rarely detected in pigs
beyond the first week of infection (Van Reeth et al., 2012) whereas oral fluid antibody was
for ≥ 42 days post inoculation and correlated with serum antibody (r ~0.80) (Chapter 3 and
4). Notably, the IAV oral fluid nucleoprotein (NP) antibody assay described in this
dissertation demonstrated the capacity to detect multiple IAV subtypes. Specifically, no
difference was found in the detection of oral fluid NP antibody between H1N1 and H3N2
inoculated pigs (Chapter 3 and 4). This is an example of a screening assay based on the
detection of antibody targeting conserved elements of the virus, in this case the viral
nucleoprotein (NP). Indeed, NP is so highly conserved among IAV subtypes that antibody
assays targeting this protein can detect IAV infections across subtypes, as well as host
species.

Future research should continue to explore diagnostic applications of oral fluid for the
purpose of improving IAV surveillance. Areas of investigation should include the evaluation
of other IAV conserved elements, e.g., nonstructural proteins (NS) and matrix protein (M)
for “universal” IAV antibody detection and for their use in differential vaccines and
diagnostic assays based on the presence (positive DIVA) or absence (negative DIVA) of
specific protein(s) in the vaccine. Indeed, several different DIVA strategies have been
proposed for surveillance of avian influenza (Suarez, 2005) and some of them could
potentially be used in swine. These would include subunit vaccines targeted to the
hemagglutinin (HA) or neuraminidase (NA) proteins or vaccines missing specific internal
viral proteins, e.g., matrix (M), nonstructural protein 1 (NS1), or NP. Concurrent
development of the vaccine and oral fluid diagnostic assays could provide a powerful tool for
the control IAV in swine population by facilitating a method to effectively identify infected
populations. Overall, it is important that research on the diagnostic applications of oral fluid
continues because of its potential to provide the ecologic information necessary to achieve an
effective control strategy for IAV in swine.
REFERENCES


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