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Considerations for improved surveillance of Influenzavirus A in swine populations

Christa Kalberer Goodell
Iowa State University

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Considerations for improved surveillance of *Influenzavirus A* in swine populations

by

Christa Kalberer Goodell

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
Jim Roth
Rodger Main
Montserrat Torremorell
Chong Wang

Iowa State University
Ames, Iowa
2013

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DEDICATION

The completion of this dissertation is solely due to the unrelenting support and confidence of two individuals: my steadfast, selfless, loving husband and soul mate, Jeffrey, and my mentor, colleague and friend, my major professor, Dr. Jeff Zimmerman.

For my children, who have endured many sacrifices, thank you for bearing with me throughout my graduate program and for understanding that, although time consuming, this work has allowed us new opportunities. I hope this achievement demonstrates to you that commitment to and accomplishment of goals will gain you success throughout life.
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ABSTRACT

Influenza A virus (IAV), a member of the Orthomyxoviridae family, is a zoonotic agent with worldwide implications for health in humans, pigs and poultry (Van Reeth et al., 2012). The modern history of IAV began with the 1918 human pandemic (Spanish flu) and the concurrent appearance of a previously unrecognized disease in pigs. Dr. J.S. Koen, a Bureau of Animal Industry veterinarian, termed the pig epidemic "hog flu" because of its clinical similarity to Spanish flu in humans (Dorset et al., 1922). This concept was echoed by Shope in subsequent experiments (1931a) and a 1958 publication in which he stated:

"Antigenically, the swine influenza virus is closely related to the type A human influenza virus; and, in fact, had it initially been isolated from man instead of swine, it would have been classed as a typical type A human influenza virus" (Shope, 1958).

In North America, a highly stable H1N1 influenza virus was the dominant strain in swine until the appearance in the 1990s of a variety of reassortant viruses containing gene segments from both humans and swine (double reassortant) (Vincent et al., 2008). Subsequent sequencing of clinical isolates from Midwestern US swine identified additional genetic complexity, i.e., triple reassortant viruses containing genes from humans, pigs and birds (Vincent et al., 2008). This escalating diversity was the result of on-going genetic and antigenic change among influenza viruses evolving to maintain competitive viability (Domingo et al., 1996). For those responsible for animal health, the diversity of influenza A viruses has compounded the challenge of prevention and control in swine populations and complicated the process of diagnostic testing in the laboratory.

Once considered a seasonal infection with rapid onset and recovery (Shope, 1931b), influenza A virus is now considered a major component of the "porcine respiratory disease complex" and a significant pathogen of pigs in its own right (Van Reeth et al., 2012). Holtcamp, et al. (2007a, b) identified influenza A virus as one of the most economically significant pathogens at all stages of production: breeding, nursery, and finishing populations. In fact, in a survey of practitioners, it ranked equal to porcine reproductive and
respiratory syndrome virus (PRRSV) in health impact and only second to PRRSV in productivity losses.

The public health significance of influenza A virus in swine is also not to be discounted. In the 2009 H1N1 influenza pandemic, an event in which pigs only played a bystander role, 17 countries closed their doors to U.S. pork or pork products and the market experienced an immediate drop in U.S. consumer demand for pork. Final estimates are not available, but losses to U.S. producers between April 24 and December 31 2009 were estimated at $1.3 billion (Pappaioanou and Gramer, 2010). Preparing to deal with events such as these before they occur is the responsible thing to do. Thus, the objective of the work presented in this dissertation was to explore critical parameters relevant to the development of an effective and sustainable surveillance system for influenza A viruses.

Chapter 1 describes considerations for creating an efficient, responsive, and robust surveillance system for exotic and endemic diseases, e.g., influenza A virus, of importance to North American swine producers and includes a discussion of the historical changes in the industry that make a new approach necessary.

Chapter 2 addresses the repeatability and reproducibility of current influenza A virus oral fluid diagnostic assays. The study used a ring test format to assess the performance of real-time, reverse-transcriptase polymerase chain reaction (rRT-PCR) and virus isolation assays for oral fluid specimens in eight participating laboratories. For rRT-PCRs, cycle threshold (Ct) increased consistently with dilution, but exhibited wide variation. As a consequence it was not possible to predict virus isolation success based on Ct values. Recovery of virus was inversely related to the dilution of the sample and was generally unsuccessful at lower virus concentrations. Most significantly, the ring test identified repeatability issues for both rRT-PCR and VI assays within certain laboratories and deficits in reproducibility between laboratories.
Chapter 3 followed the pattern of influenza A virus shedding in pen-based oral fluid and individual nasal swabs collected from pigs inoculated with subtypes H1N1 or H3N2 under experimental conditions. This work confirmed that the detection of influenza A virus was significantly affected by pig vaccination status \( (p < 0.0001) \), time post inoculation \( (p < 0.0001) \), and specimen-assay interaction \( (p < 0.0001) \), but not virus subtype \( (p = 0.89) \). Vaccination and/or increasing time post inoculation reduced the estimated probability of detection for all assays: rRT-PCR, virus isolation, and a rapid point-of-care (POC) assay. Virus isolation was more successful using nasal swabs than oral fluids, but both virus isolation and detection by point-of-care testing were generally unsuccessful later than 6 days after inoculation. Overall, the combination of oral fluid and rRT-PCR testing offered the highest diagnostic sensitivity in both vaccinated and unvaccinated animals.

The work described in Chapter 4 examined the performance of influenza A virus serum antibody assays using the samples collected during the experiment described Chapter 3. Assays compared in the study included commercial ELISAs and hemagglutination inhibition (HI) assays. The homologous HIs showed 100% diagnostic sensitivity, but largely failed to detect infection with the heterologous virus. Similarly, the commercial H1N1- and H3N2-specific indirect ELISAs were ineffective at detecting influenza A virus antibodies in swine infected with the contemporary influenza viruses used in the study. However, a commercial nucleoprotein (NP) antibody blocking ELISA assay demonstrated a diagnostic sensitivity and specificity of 95.5% and 99.6%, respectively, at sample-to-negative (S:N) cut-off of \( \leq 0.60 \). Statistically significant factors that affected S:N responses included vaccination status, inoculum (virus subtype), time post inoculation, and interactions between those factors \( (p < 0.0001) \). These results suggest that NP serum antibodies could provide an ideal universal diagnostic screening target and facilitate a cost-effective approach for the detection and surveillance of influenza A virus infections in swine populations.
REFERENCES


Dissertation Organization

This thesis consists of 4 chapters. Chapter 1 is a general introduction and road map for contemporary swine surveillance programs, “Protecting our future - A road map for practical, real-time, on-farm infectious disease surveillance”. Chapter 2, “Ring test evaluation of the detection of influenza A virus in swine oral fluids by real-time, reverse transcription polymerase chain reaction (rRT-PCR) and virus isolation” has been submitted for publication in Preventive Veterinary Medicine. Chapter 3, “Probability of the detection of influenza A virus subtypes H1N1 and H3N2 in individual nasal swab and pen-based oral fluid specimens from pigs over time” has been submitted for publication to Veterinary Microbiology. Chapter 4, "Evaluation of screening assays for the detection of Influenza A virus serum antibodies in swine” has been submitted for publication in Preventive Veterinary Medicine. References, tables, and figures for each manuscript follow each discussion section respectively. The last chapter contains general conclusions of this dissertation.
CHAPTER 1. PROTECTING OUR FUTURE - A ROAD MAP FOR PRACTICAL, REAL-TIME, ON-FARM INFECTIOUS DISEASE SURVEILLANCE

A manuscript to be submitted to the Journal of Swine Health and Production

C Goodell, C Wang, R Main, JA Roth, P Sundberg, L. Wagstrom, P Webb, J Zimmerman

SUMMARY

Sweeping changes during the 20th century metamorphosed pig production from small, extensive (outdoor), labor-dependent enterprises into intensive (confined), capital-dependent, production systems. These changes produced safe, wholesome, inexpensive food for the North American consumer and pork products capable of competing successfully in international markets, e.g., U.S. pork exports in 2012 were valued at $6.3 billion (USD). However, the same infrastructure and production characteristics that led to improved efficiencies, i.e., larger populations with high throughput and extensive pig movement between sites, have left the industry highly vulnerable to the rapid dissemination of infectious diseases, with foreign animal diseases (FAD) of particular concern. In this commentary we review the evolution of the industry and the rationale for a new approach to the control of infectious diseases. We suggest that real-time, on-farm, producer-driven surveillance is necessary to provide producers the means to control endemic infectious agents and prepare for the rapid identification, control, and elimination of a foreign animal disease in the event of its introduction. Further, we outline a plan whereby real-time surveillance could be achieved using a decentralized approach based on (1) on-farm sample collection, (2) diagnostic testing in accredited non-Federal laboratories, and (3) continuous analysis and response to test results.
COMMENTARY

The purpose of this commentary is to review the historical development of swine production in the U.S. and begin the discussion of a plan for protecting the future of the industry. The premise of the argument is that the structure of the industry and the lack of timely surveillance information make rapid and effective response to infectious diseases (endemic and exotic) impossible. While surveillance does exist at present, e.g., submissions to diagnostic laboratories, cull and terminal markets (http://www.aphis.usda.gov/animal_health) we believe that producer-driven, on-farm sampling is the best method to control endemic infectious agents and rapidly identify, control, and eliminate a foreign animal disease in the event of its introduction.

U.S. swine inventory numbers are available as far back as 1850. For most of this period, but particularly between 1890 and the present, the total number of pigs in the U.S. remained at 50 to 60 million animals (USDA, 2008). In contrast, the total number of swine farms has declined steadily since 1900; a pattern that continues into the present (MacDonald and McBride, 2009; USDA, 2008).

Historically, improvements in productivity were based on breeding for selected characteristics and phenotypic traits. Beginning with the formation of the U.S. Bureau of Animal Industry (1884) and continuing into the 20th century, the scientific method was applied to research in swine genetics, nutrition, reproduction, housing, and disease prevention / control. Active efforts at controlling the impact of disease on productivity began when researchers started identifying pathogens and devising methods of control. An early example was the "McLean County system of swine sanitation", the creation of the brilliant U.S. Bureau of Animal Industry parasitologist, B.H. Ransom. First tested in 1919, it was based on Ransom's discoveries of parasite life cycles and was designed to work by breaking the cycle of parasite transmission between the sow and her offspring using basic sanitation procedures. When field tested, it markedly reduced parasite burden and improved pig survivability, growth, and productivity. A 20% improvement to overall pig production was noted in farms implementing the strategy. Practical and simple, the McLean County system
was rapidly adopted by producers (Anon, 1926). Improvements in housing and environment continued through the 20th century, but the wholesale conversion of extensive production into intensive production in the 1970s and 1980s resulted in sophisticated, highly-engineered, capital-intense facilities that reduced manual labor, increased efficiency, and allowed for housing larger populations. Thus, 50% of U.S. hogs were produced in 214 counties in 1982, but only 81 counties in 2007 (O’Donoghue et al., 2011).

Cumulatively, these efforts have resulted in remarkable improvements in pork production (Table 1), e.g., productivity increased from 4,600 pounds of pork per sow per year in 1996 to 5,200 pounds in 2005 (Vansickle, 2006). Key and McBride (2007) compared industry-average production costs for hogs in 1992, 1998, and 2004. In the feeder-to-finish sector, average feed conversion rates (pounds of feed per 100 pounds of gain) fell from 383 pounds in 1992 to 214 pounds in 2004 and labor hundredweight of gain fell from 53 minutes to 9 minutes. As a result, average production costs fell from $37.54 per hundredweight in 1992 to $26.59 in 2004, despite increases in the cost of capital, labor, feed, and other inputs. In real terms, industry-wide average costs dropped by 44 percent between 1992 and 2004.

Consistent with historic trends, the total inventory of the U.S. swine herd in December 2012 was at 66.3 million head, including 5.82 million breeding animals and a market hog inventory of 60.5 million (NASS, 2012a). Measures of productivity included an industry average of 10.15 pigs weaned per litter for the last quarter of 2012 (NASS, 2012a), with some herds achieving 11.45 pigs per litter (PigCHAMP, 2012). In addition to pigs farrowed in the U.S., the 2012 inventory included ~6,000,000 live pigs, imported chiefly from Canada (ERS, 2013). Most (~85%) of the Canadian imports consisted of feeder or weaner pigs shipped to grow-out facilities in the Corn Belt (Plain and Brown, 2013).

The productivity and economics of the pork industry are staggering. In 2007, the industry produced 21 billion pounds of pork with a retail value of $51.7 billion (Lowe and Gereffi, 2008). In 2011, pork production added $34.5 billion to the U.S. gross national product
International markets have become increasingly important to the industry, especially in the last 10 years (Table 2). In 1990, exports represented less than 1.6% of U.S. pork production (Brester et al., 1997). Following two decades of continuous growth, exports in 2012 represented 27% of production and were valued at $6.3 billion (USD). Export of live animals also provides revenue, but on a relatively small scale. In 2012, 55,059 live pigs were exported from the U.S., most as market animals to Mexico and breeding stock to China (USDA, 2013).

While immensely productive, the continued health and economic viability of the U.S. swine herd is highly vulnerable. In large part, this is a consequence of the production practices that created and sustain it. Over time, pork production has become concentrated into fewer farms with more pigs per farm, e.g., Iowa farms averaged a total inventory of 250 animals in 1980 and 1,500 in 2002 (Flora et al., 2007). Equally important for its effect on disease spread is the extensive movement of pigs between premises. A relatively new industry practice, large numbers of young pigs are moved into feeding operations located in the Corn Belt because it is cheaper to move young pigs than to transport feedstuffs to the pigs (Table 3).

An important, but unanticipated, result of these changes was to tilt the balance in favor of infectious diseases. Larger pig populations favor pathogens because herd immunity becomes increasingly fragile as population size increases (Evans et al., 2010). The movement of live animals provides the means for pathogens to reach geographically distant populations. While challenging, these conditions are not without historical precedent. A similar pattern was observed as humans moved from farms and began to congregate in cities. With minimal editing, Rosner's (2010) description of human health in the U.S. during the latter part of the 18th century could describe the present conditions in swine health: "Epidemic disease, once a local phenomenon circumscribed by the relative lack of mobility among self-sufficient and isolated rural communities, began to sweep through the nation along the well-established trade routes as the nation's boundaries and population expanded ...." Of course, the human health issues of the 18th century were resolved in the 19th and 20th centuries through better understanding and management of the factors that impacted health. Equipped with better
science than our predecessors, we can certainly resolve these issues in swine health. The motive for improving the current situation is both to assure animal health and to protect a valuable asset. There is every reason for pork producers to anticipate a need to meet increasing animal health expectations from retailers and consumers, e.g., McDonald's Agricultural Assurance Programme (Kaeppel, 2005). Of equal concern for the industry, the detection of a foreign animal disease (FAD), e.g., African swine fever virus, classical swine fever virus, or foot-and-mouth disease virus, anywhere in North America will have severe economic consequences. Rendleman and Spinelli (1999) estimated the cost of an outbreak of African swine fever virus in the U.S. at $4,500,000,000 to $5,445,000,000 in 1992 dollars. Of course, the 1992 estimate could not have anticipated the current value of exports or the consequences of an FAD-mandated "stop movement" order. While the appearance of an FAD represents the worst possible event, equally worthy of consideration are the perpetual industry losses to endemic diseases and our failure to bring them under control, the classic example being porcine reproductive and respiratory syndrome virus (PRRSV), which costs U.S. producers $664,000,000 annually (Holtkamp et al., 2013).

In 2001, the Animal Health Safeguarding Review (NASDA, 2001) noted that, "Infrastructure inadequacies, especially in terms of staffing and facilities, are now so deep that the [USDA APHIS] system cannot appropriately respond to a severe animal health crisis." In light of this, the swine industry needs to lead the development of a surveillance system capable of providing accurate, real-time data on infectious diseases in the U.S. swine herd. If an effective national surveillance system is to be achieved, it will be driven by industry and coordinated with government. The decentralized approach we propose is based on:

1. On-farm sample collection by farm personnel and veterinarians.
2. Routine testing and reporting by accredited non-Federal laboratories, e.g., National Animal Health Laboratory Network (NAHLN) laboratories.
3. Electronic reporting of test results to producers and veterinarians, with access restricted to designated users.
On-farm sampling  Serum samples have historically been used as ante mortem surveillance samples, e.g., the U.S. pseudorabies eradication effort (Anderson et al., 2008), but oral fluid samples are also a viable option. A relatively new sample type in swine diagnostics, the Iowa State University Veterinary Diagnostic Laboratory performed 10,329 tests on swine oral fluids in 2010, 32,517 in 2011, and 60,192 in 2012, i.e., producers and veterinarians have adopted this approach. Oral fluid specimens offer specific advantages for surveillance: (1) they are easily collected by a single designated person; (2) samples can be collected frequently and easily without stress to pigs or people; and (3) they provide a higher probability of analyte detection with fewer samples than serum (Olsen et al., 2013). The disadvantage of oral fluids at the present time is that a full complement of diagnostic assays has not yet been developed.

Theoretically, either specimen type (serum or oral fluid) would be suitable for nucleic acid (PCR) or antibody-based testing. Testing flexibility is desirable because, in the event of uncertainty, it allows for follow-up testing using different technologies.

Each diagnostic methodology presents advantages and disadvantages. The detection of nucleic acid reflects the circulation of pathogens in the present; an important issue from the point of view of timeliness. However, nucleic acid assays are more expensive than antibody-based assays and test performance has been an issue (Fetzer et al., 2006; Goodell, et al., 2013; Truyen et al., 2006). Antibody is abundant in serum, readily detected in oral fluid (Prickett and Zimmerman, 2010), and the cost of antibody assays is significantly lower than PCR-based assays. In contrast to nucleic acid, antibody provides a prolonged window of detection because it reflects both recent and past exposure history. At the farm level, antibody assays are compatible with continuous monitoring of population immune status using a control-chart approach. On the other hand, point-in-time detection of pathogens or nucleic acid may be useful for pathogen characterization and/or vaccine development. Ultimately, the selection of which approach to use should be dictated by the purpose of testing.
Ante mortem sampling on the farm is the best method of acquiring accurate, real-time health information and, whether based on serum or oral fluid collection, is consistent with sampling procedures used on most farms. Sample size, sample frequency, and sample allocation on the farm are key facets of surveillance. "Representative sampling" was first proposed in 1895 and applied to disease detection in swine herds in the 1970's as an alternative to whole-herd testing (Cannon and Roe, 1982; Kruskal and Mosteller, 1980; USDA 2008). While appropriate for the small herds of the 1980's, these procedures are inadequate for large, complex, production systems. New, statistically valid guidelines for serum and oral fluid sampling (sample size, sample allocation, and sample frequency) must be developed for contemporary use. Importantly, in the event of a FAD, sample size and frequency could easily and quickly be adjusted upward ("dialed up") to increase herd-level sensitivity - without the need to change any other part of the process.

Routine testing The National Animal Health Laboratory Network (NAHLN) was initiated in 2002 in response to the USDA Safeguarding Review. It was envisioned as the core of the U.S. emergency response and recovery program for foreign animal diseases and was tasked with developing the capacity for early detection, rapid response (surge capacity during an outbreak), and proof of negative status. Starting with 12 state and university veterinary diagnostic laboratories, the system has expanded to 59 laboratories in 42 States (www.aphis.usda.gov/animal_health/nahln/ accessed April 12, 2013). However, if the NAHLN laboratories are to fulfill the emergency response and recovery role originally envisioned, testing for FADs needs to be expanded and new FAD tests for oral fluid specimens developed. In addition, on-going proficiency testing for FADs in NAHLN laboratories should be implemented to provide (1) FAD assay performance assessment and technician training prior to an FAD event; (2) documentation of FAD-negative status for export partners; and (3) immediate capacity to accurately test massive numbers of samples for FADs. For example, the Iowa State University Veterinary Diagnostic Laboratory tested 5,000-to-10,000 serum samples daily during the US PRV eradication project. Far greater capacity will be needed from the moment an FAD is detected in North America. The only way to meet this anticipated need is to disseminate FAD training and testing across NAHLN
laboratories well before the emergency occurs. This does not impinge the oversight and reporting responsibilities held by State and Federal authorities. That is, FAD test result interpretation, analysis, and dissemination remain the responsibility of the USDA.

**Electronic reporting**  In the past, the collection, manipulation, and analysis of surveillance data was slow, cumbersome, and expensive. For good reason, little surveillance was done. However, today's NAHLN laboratories are "electronified", i.e., they have converted their routine testing and reporting activities from hardcopy to electronic formats. The immediate benefit of this effort was to provide faster test reporting to laboratory clients via email and/or web access. An unanticipated benefit of electronification has been development of the capacity to collect, compile, integrate, manipulate, and analyze diagnostic data within and across NAHLN laboratories. Data collection, formerly the most difficult part of surveillance, is now routine. It follows that real-time, on-farm, national surveillance could be achieved quickly and easily simply by coordinating and integrating the network of NAHLN electronic data. To some degree, this process of integration has already begun, e.g., select laboratories and their clients are using Disease BioPortal (http://fmdbioportal.ucdavis.edu). Nothing in this approach affects or diminishes client-laboratory confidentiality. Indeed, the American Association of Veterinary Laboratory Diagnosticians (AAVLD) requires that AAVLD-accredited laboratories protect client confidentiality (AAVLD, 2012). Controls to further limit user data access and protect confidential information are easily implemented.

Any attempt to remedy the current situation can only succeed if it provides direct benefit to individual producers. Inherent in this discussion is the belief that future increases in producer profitability will be derived from incremental improvements in swine population health management that result in producing pork at lower cost. Such improvements can only occur if the data are available to support rational decision-making.

An intangible benefit to producers is the potential to control future risk. OIE guidelines provide for business continuity in the face of a FAD event, but require certain elements to be in place prior to the event (OIE, 2012). For example, a production system (or a flow within a
complex production system) with a record of on-going surveillance and defined production, biosecurity, and diagnostic protocols is permitted to continue animal movement and product export - as long as on-going surveillance continues to provide evidence of the absence of the FAD.

CONCLUSIONS

The intent of this commentary was to provide a historical perspective on the progression of events that led to the development of the contemporary U.S. pork industry. While highly productive, the current structure leaves the industry extremely vulnerable to infectious diseases. We have briefly discussed and outlined the salient features of a practical, effective surveillance system that could directly benefit the industry by providing the data needed to improve the control of endemic diseases at the herd level and eliminate exotic diseases in the event of their introduction. We acknowledge the novelty of the plan, but counter that the combination of Necessity and New Technology has created the opportunity to do things in new ways. Myriad details are lacking in the outline, but we believe these will emerge with input from of pork producers and swine health specialists as the discussion moves forward.
REFERENCES


Plain R and Brown S. Hog outlook: 2012 pork exports were record high.


Table 1. Commercial U.S. hogs marketed from selected years*

<table>
<thead>
<tr>
<th>Year</th>
<th>Hogs marketed (head)</th>
<th>Pork produced (pounds)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1970</td>
<td>85,817,000</td>
<td>14,699,000,000</td>
</tr>
<tr>
<td>1980</td>
<td>96,074,000</td>
<td>16,617,000,000</td>
</tr>
<tr>
<td>1990</td>
<td>85,135,000</td>
<td>15,354,000,000</td>
</tr>
<tr>
<td>2000</td>
<td>97,976,000</td>
<td>18,952,000,000</td>
</tr>
<tr>
<td>2007</td>
<td>109,172,000</td>
<td>21,963,000,000</td>
</tr>
<tr>
<td>2011</td>
<td>110,862,000</td>
<td>22,779,000,000</td>
</tr>
</tbody>
</table>

*(NPB, 2011 Quick Facts from Livestock slaughter and Poultry Slaughter, 2009-2011)
Table 2. Total U.S. pork exports (2003-2012)*

<table>
<thead>
<tr>
<th>Year</th>
<th>Metric Tons Exported</th>
<th>Value ($USD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2003</td>
<td>757,406</td>
<td>$1,582,000,000</td>
</tr>
<tr>
<td>2004</td>
<td>1,023,413</td>
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<td>2005</td>
<td>1,157,689</td>
<td>$2,634,000,000</td>
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<tr>
<td>2006</td>
<td>1,262,499</td>
<td>$2,864,000,000</td>
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<td>2007</td>
<td>1,305,622</td>
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<td>2008</td>
<td>2,052,447</td>
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<td>2009</td>
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<td>$6,108,000,000</td>
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<tr>
<td>2012</td>
<td>2,262,109</td>
<td>$6,322,000,000</td>
</tr>
</tbody>
</table>

Table 3. Inshipments of hogs to selected U.S. states for selected years*

<table>
<thead>
<tr>
<th>Year</th>
<th>Iowa</th>
<th>Indiana</th>
<th>Minnesota</th>
<th>Illinois</th>
<th>Total U.S</th>
</tr>
</thead>
<tbody>
<tr>
<td>1980</td>
<td>1,740,000</td>
<td>549,000</td>
<td>226,000</td>
<td>510,000</td>
<td>4,628,000</td>
</tr>
<tr>
<td>1990</td>
<td>1,400,000</td>
<td>240,000</td>
<td>262,000</td>
<td>359,000</td>
<td>4,317,000</td>
</tr>
<tr>
<td>2000</td>
<td>11,600,000</td>
<td>1,050,000</td>
<td>3,150,000</td>
<td>1,470,000</td>
<td>24,514,000</td>
</tr>
<tr>
<td>2010</td>
<td>21,200,000</td>
<td>2,632,000</td>
<td>7,089,000</td>
<td>1,898,000</td>
<td>39,571,000</td>
</tr>
<tr>
<td>2011</td>
<td>22,500,000</td>
<td>2,681,000</td>
<td>6,300,000</td>
<td>2,261,000</td>
<td>39,812,300</td>
</tr>
</tbody>
</table>

*From Haley (2004) and NASS (2012b).
CHAPTER 2. RING TEST EVALUATION OF THE DETECTION OF INFLUENZA A VIRUS IN SWINE ORAL FLUIDS BY REAL-TIME, REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION AND VIRUS ISOLATION


A paper submitted to Preventive Veterinary Medicine

ABSTRACT

Successful swine health monitoring and disease surveillance depends on reproducible diagnostic assays to support good decisions. To evaluate assay performance for swine oral fluids (OF), the probability of detecting influenza A virus (IAV) in OF specimens was calculated for each of 13 real-time, reverse transcription polymerase chain reaction (rRT-PCR) and 7 virus isolation (VI) assays. To conduct the study, OF was inoculated with contemporary H1N1 or H3N2 IAV and serially 10-fold diluted (10^-1 to 10^-8). In total, 8 participating laboratories received 180 randomized samples, i.e., 10 replicates of each subtype at each dilution and 20 IAV-negative oral fluid samples, and performed the IAV rRT-PCR and VI procedure(s) of their choice. Analysis of the results using a mixed-effect logistic regression model identified the variables significant to IAV detection in OF by rRT-PCR or VI as dilution (p <0.0001) and assay (p <0.0001). Virus subtype was not significant to IAV detection by rRT-PCR (p = 0.4566) or VI (p = 0.1006). Pairwise comparisons of assay performance identified significant differences in detection for both rRT-PCRs and VIs. For rRT-PCRs, Cts increased consistently with dilution, but exhibited wide variation. As a consequence it was not possible to predict VI success based on Ct values. VI was inversely related to the dilution of the sample and was generally unsuccessful at lower virus concentrations. Whereas both routine diagnostics and programmatic surveillance require tests that provide accurate, consistent performance, significant differences in reproducibility were observed among the assays evaluated.
INTRODUCTION

Influenza A virus (IAV) causes morbidity, mortality and economic losses at all stages of pig production, particularly when infection occurs concurrently with other respiratory pathogens, e.g., porcine reproductive and respiratory syndrome virus (PRRSV). In addition, IAV is zoonotic and moves relatively easily between pigs and people. There are well-recognized pig-to-human transmission events, e.g., outbreaks at county fairs in Indiana (Richards et al., 2012) and Ohio (Killian et al., 2012), but viruses containing human-origin genes have also played a role in clinical influenza in swine (Nelson et al., 2012). In the U.S., influenza virus infections in humans and swine are monitored by the Centers for Disease Control and Prevention (United States Department of Health and Human Services, 2012) and the United States Department of Agriculture (USDA, 2010). This effort tracks the regional distribution and genetic changes in viral subtypes circulating in human and swine populations. In pigs, current ante mortem surveillance is based on individual animal sampling (nasal swabs), although the short duration of shedding in nasal secretions limits the probability of virus isolation (VI) or detection by real-time reverse transcription polymerase chain reaction (rRT-PCR) after day 6 and day 8 of infection, respectively (Goodell et al., 2013). This window of detection is reduced in vaccinated animals to 4 and 5 days post infection for VI and rRT-PCR, respectively (Goodell et al., 2013). As an alternative to individual pig samples, IAV may be detected in pen-based oral fluid (OF) specimens by rRT-PCR for at least 14 days post inoculation (DPI) in unvaccinated animals and at least 7 DPI in vaccinated animals (Goodell et al., 2013). For ease of sampling and improved IAV detections rates in swine populations, OF is gaining favor in surveillance programs (D. Pyburn, personal communication).

Regardless of specimen type, successful swine health monitoring and disease surveillance is dependent on reliable diagnostic assays to support good decisions. A variety of VI procedures, as well as commercial and in-house IAV rRT-PCR assays, are currently in place in veterinary diagnostic laboratories, but no direct comparisons of assay performance have been performed on swine specimens. Although proficiency panels are useful in test validation and harmonization (Belak and Thorén, 2008; Holden et al., 2011; Suarez et al., 2007), they also serve to assess competency, supplement laboratory quality control measures,
and improve uniformity in assay performance (Suarez et al. 2007; Valentine-Thon et al. 2001; Van Vliet et al., 2001; Yen-Lieberman et al. 1996). For example, in follow-up performance studies where identical protocols, reagents, and/or training were provided, greater assay reproducibility and repeatability was observed (Paton et al., 2000b; Slomka et al., 2007; Spackman and Suarez, 2005). Ring tests are proficiency tests coordinated among multiple collaborating laboratories (Duarte et al., 2007). Inclusion of sufficient numbers of replicates and dilutions allow for statistical analysis of assay reproducibility (between laboratory) and repeatability (within laboratory) and facilitate process improvement. In this study, the objectives were to evaluate IAV assays for OF, determine the correlation between rRT-PCR results and the probability of successful VI, and identify the most accurate rRT-PCR and VI protocols using a ring test design.

MATERIALS AND METHODS

Experimental design
The probability of detecting IAV in swine oral fluid was calculated for each of 13 rRT-PCRs and 7 VI assays performed at 8 laboratories. To conduct the study, swine oral fluid was inoculated with H1N1 or H3N2 IAV and serially 10-fold diluted ($10^{-1}$ to $10^{-8}$). The complete sample set consisted of 180 randomized samples, i.e., 10 replicates of each subtype at each dilution and 20 IAV-negative oral fluid samples. A mixed-effect repeated measures logistic regression model was used to determine the association between the detection of IAV and variables of interest, i.e., IAV subtype, dilution, assay, and interactions.

Ring test samples
The OF used in the ring test was collected from four sows in isolation at the Iowa State University Livestock Infectious Disease isolation Facility (IACUC # 11-09-6832-S). To verify the health status of the sows prior to OF collection, serum was tested at the Iowa State University Veterinary Diagnostic Laboratory for evidence of infection with porcine reproductive and respiratory syndrome virus (Eshelman, 2010), *Mycoplasma hyopneumoniae* and IAV using established laboratory procedures (Boesenberg, 2010). OF was collected from these animals daily for 28 days using cotton ropes until a total of 5.4 L
was accumulated (Prickett et al., 2008). At the end of each daily collection, OF was centrifuged (13,000 x g) for 10 min and stored in 50 ml tubes at -80°C.

To prepare ring test samples, OF was thawed, aggregated in a sterile 6 L flask and mixed on a magnetic stir plate for 1 hr in a biosafety cabinet. Negative control samples were generated by dispensing 2.6 ml aliquots of aggregated OF into 3 ml cryovials. Thereafter, subtype-specific IAV samples were prepared from stock solutions of A/Swine/Ohio/511445/2007 γ H1N1 virus and /Swine/Illinois/02907/2009 Cluster IV H3N2 virus. Viruses had been propagated on Madin-Darby Canine Kidney cells to a virus concentration of 1 x 10^6.5 median tissue culture infectious dose (TCID_{50}) per ml. From these virus stock solutions, 10-fold dilutions (10^{-1} to 10^{-8}) of H1N1 and H3N2 were created and dispensed as 2.6 ml aliquots into 3 ml cryovials. One ml of each stock solution was retained for back titration.

Eleven sample sets composed of 20 negative and 160 IAV-inoculated aliquots, i.e., 10 replicates of each IAV subtype at each dilution (10^{-1} through 10^{-8}) were prepared for distribution to participating laboratories. Each aliquot was identified by "set number" and a sample (random) number between 1 and 180. Sample sets were sorted by random number, stored at -80°C, and then shipped overnight on dry ice to the 8 participating laboratories. Temperature indicators were placed in each box (13 x 13 cm) of cryovials (60 samples) to detect exposure of the contents to temperatures ≥-18°C during the shipment process. Participants confirmed that samples were still frozen and boxes contained dry ice at the time of arrival. Samples were immediately returned to -80°C conditions until tested at the recipient laboratory.

All laboratories invited to participate had prior experience with molecular techniques for the detection of IAV in swine specimens including nasal swabs, tissue and oral fluid. All invited laboratories agreed to participate. Each laboratory was at liberty to perform the IAV rRT-PCR and VI procedure(s) of their choice. Among the 8 laboratories, 5 tested the samples using one rRT-PCR procedure, 2 laboratories performed 2 procedures, and one laboratory
performed 4. Six laboratories performed VI; one of which used two different VI methods. Testing results were reported by sample number as binary (yes/no) outcomes with cycle threshold (Ct) values reported for rRT-PCR-positive samples.

**Data analysis**

A mixed-effect repeated measures logistic regression model was used to analyze the binary responses: detection of IAV in oral fluid by rRT-PCR (yes/no) and VI (yes/no). The explanatory variables of interest include dilution (10^{-1} through 10^{-8}), virus subtype (H1N1 or H3N2), assay (VI 1-7 or rRT-PCR 1-13), and relevant interactions, with sample as a random effect. Fixed effects were considered significant at α = 0.05. The degree of variability among all aspects of assay protocols precluded statistical analyses of the individual components. Pairwise differences between assays were assessed using Tukey’s t-tests. The probability of detecting IAV in OF by dilution was estimated for rRT-PCR and VI tests from the mixed-effect repeated measures logistic regression model. The model was also used to estimate the detection dose 50 (DD_{50}) for each assay, i.e., the inoculum dilution at which there was a 50% probability of detection.

**RESULTS**

Back titration of the original virus inocula estimated the initial concentrations at 1 \times 10^{7.5} TCID_{50}/ml and 1 \times 10^{6.57} TCID_{50}/ml, respectively, for the A/Swine/Ohio/511445/2007 γ H1N1 and A/Swine/Illinois/02907/2009 Cluster IV H3N2 viruses.

Table 1 lists all rRT-PCR testing results by assay (PCRs 1-13). Eleven of 13 assays reported at least 1 false positive among the 20 negative samples. All assays detected virus in all H1N1-inoculated samples through the 10^{-3} dilution; after which detection decreased by assay and dilution. Among H3N2-inoculated samples, all assays detected virus in 10 of 10 samples at 10^{-1} dilution and 12 of 13 assays detected virus in 10 of 10 samples at both 10^{-2} and 10^{-3} dilutions. Detection decreased by assay and dilution thereafter. Mean Ct-values by dilution varied widely among assays, although Ct-values increased consistently within each assay as the dilution increased (Figure 1).
Table 2 shows all VI results by assay (VIs 1-7). Successful isolation was inversely related to the dilution of the sample. One assay reported a single false positive VI among the 20 negative samples. Six of the 7 assays detected virus in all H1N1- and H3N2-inoculated samples at 10^{-1} dilution. VI decreased by assay and dilution thereafter.

Analysis of the data in a mixed-effect logistic regression model identified the variables significant to IAV detection in oral fluid by rRT-PCR or VI as dilution (p <0.0001) and assay (p <0.0001). Virus subtype was not significant to IAV detection by rRT-PCR (p = 0.4566) or VI (p = 0.1006). The interaction between dilution and subtype was also not significant for detection by either rRT-PCR or VI (p = 0.7568 and p = 0.0658, respectively). Therefore, virus subtype was subsumed into a single variable for subsequent analyses.

The probability (Pr) of IAV detection over time was calculated using Equations 1 and 2.

\[ \lambda = \text{Logit}(P(X)) = \alpha + \beta_1 X_1 + \beta_2 X_2 \quad \text{Equation 1} \]
\[ \alpha = \text{intercept} \]
\[ \beta_1 = \text{regression coefficient for concentration} \]
\[ \beta_2 = \text{regression coefficients for assay procedure} \]

\[ \text{Probability (Pr)} = \frac{e^{(\lambda)}}{1 + e^{(\lambda)}} \quad \text{Equation 2} \]

The probability of detecting IAV by rRT-PCR as a function of dilution is shown in Figure 2. Notably, there was a 10^3 range in DD_{50} between the lowest and highest performing assays, i.e. 1 \times 10^{-4.68} versus 1 \times 10^{-6.68}. Pairwise comparisons among the 13 assays identified significantly different levels of detection performance. PCRs 1, 2, and 3 exhibited the highest performance and were statistically equivalent. Protocols for PCRs 1, 2, and 3 are given in Appendix A.

The probability of isolating IAV from OF as a function of dilution is given in Figure 3.
Significantly different levels of performance were identified between VI assays, with a $10^{2.67}$ range in DD$_{50}$ between the lowest and highest performing assays, i.e. $1 \times 10^{-1.41}$ versus $1 \times 10^{-4.08}$. VI was generally unsuccessful at dilutions $> 10^{-4}$. Pairwise comparisons among the 7 assays identified statistically different levels of performance. VIs 1 and 2 showed the highest performance and were statistically equivalent. Protocols for VIs 1 and 2 are reported in Appendix A.

An evaluation of the relationship between rRT-PCR Ct values and VI positivity determined that the probability of a positive rRT-PCR had no association with the probability of VI success within the same laboratory with the exception of the dilutions with the highest concentrations of virus. This absence of an association resulted from the variability in both PCR Ct values and VI performance within laboratories.

**DISCUSSION**

The use of oral fluid specimens for the detection of IAV infections in swine is a relatively recent innovation (Detmer et al., 2011; Goodell et al., 2013; Romagosa et al., 2012), but is compatible with previous reports on the use of oral fluid specimens in human and veterinary diagnostics (Prickett and Zimmerman, 2010). In particular, pen-based oral fluid specimens provide the advantage of a higher probability of disease detection in populations compared to samples collected from individual animals (Goodell et al., 2013; Olsen et al., 2013; Ramirez et al., 2012; Romagosa et al., 2012). For example, a recent study (Romagosa et al., 2012) reported that the probability of detecting IAV in oral fluid by rRT-PCR was 69% when the prevalence of infected pigs in a pen was 9% and 99% when the prevalence was $\geq 10\%$.

The primary aim of this study was to evaluate the performance of rRT-PCR and VI assays for the detection of IAV in swine oral fluid specimens. This was achieved by analyzing and ranking 13 rRT-PCR and 7 VI assays using results from identical sets of samples tested at 8 laboratories. The analysis identified marked differences in rRT-PCR and VI performance and a general lack of reproducibility among participating laboratories. These results were compatible with previous ring test studies in which a high degree of variability was observed.
among diagnostic laboratories performing similar tests (Harding et al., 2009; MacKay et al., 2008; Paton et al., 2000a; Truyen et al., 2006; Wernike et al., 2012).

Although a recent publication compared MDCK cells versus embryonated eggs for isolation of IAV (Bowman et al., 2013), no IAV VI ring tests were found in the literature. Thus, there are no reports with which to compare the VI DD$_{50}$ estimates reported in this study ($10^{-1.4}$ to $10^{-4.1}$). Compared to VI, the DD$_{50}$ for the rRT-PCR$s$s ranged from $10^{-4.68}$ to $10^{-6.68}$, confirming that PCR is a more analytically sensitive test. Although mean Cts (Figure 1) and the number of rRT-PCR positive samples (Table 1) correlated with the concentration of IAV in oral fluid, the range of Cts within dilutions demonstrated a substantial lack of precision. As a consequence, while samples with higher virus concentration had a higher probability of detection by both rRT-PCR and VI, it was not possible to predict VI success based on Ct values.

A second aim of this study was to identify specific assay or laboratory factors associated with IAV detection in swine oral fluid specimens. Although the analysis disclosed a large range in detection limits for both rRT-PCR and VI assays, i.e., a ≥ 100-fold difference in the DD$_{50}$s, the variability in equipment, conditions, reagents, and protocols precluded statistical analysis that might have identified specific variables positively (or negatively) associated with detection.

A possible weakness of the study was the uniformity of the oral fluid specimens, i.e., the oral fluid used in this study was from four sows housed under experimental conditions, rather than field samples. However, this approach assured uniformity among samples and made it possible to generate the large number of samples (n = 1980) required for statistical analyses. In addition, there is no documented difference between experimental and field samples in terms of assay performance.

PCR-based assays have played an increasing role in routine diagnostics because their diagnostic and analytical sensitivities were considered superior to virus isolation while also providing for high through-put. However, issues with PCR reproducibility and repeatability
were recognized soon after the method was introduced (Valentine-Thon, 2002). To assure minimum standards of PCR performance for high-consequence human pathogens, the European Union (EU) implemented proficiency testing of laboratories handling human specimens (Valentine-Thon, 2002). Subsequent developments in PCR technology, e.g., single tube reactions and robotic automation, have resulted in improvements that have been documented in proficiency testing over the last decade (Suarez et al., 2007). Still, significant differences in assay performance remain, particularly in samples with lower target concentrations, as seen in this study and others (Harding et al 2009; Lelie et al 2002; Schirm et al, 2002; Valentine-Thon et al 2001). Both routine diagnostics and programmatic surveillance require tests that provide accurate, consistent performance - on this point there is no compromise. Achieving this goal will require a collaborative, forward thinking, multidimensional approach to quality control that includes proficiency panel and ring trial assessments.

DECLARATION OF CONFLICTING INTERESTS

The authors declare no conflicting interests with respect to their authorship or the publication of this article.

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SOURCES AND MANUFACTURERS

b. Dr. Amy Vincent, USDA National Animal Disease Center, Ames, IA.
c. Dr. Marie Gramer, University of Minnesota, St. Paul, MN
d. IDEXX PRRS X3 Ab Test., IDEXX Laboratories, Inc., Westbrook, ME.
e. IDEXX M hyo Ab Test, IDEXX Laboratories, Inc., Westbrook, ME.
f. IDEXX AI MultiS-Screen Ab Test, IDEXX Laboratories, Inc., Westbrook, ME.
g. Web Rigging Supply, Inc., Lake Barrington, IL.
h. Fisherbrand, Fisher Scientific Company LLC., Pittsburgh, PA.
j. Wheaton, Millville, NJ.
k. WarmMark® Time-Temp Tags, ShockWatch®, Graham TX.
l. QIAGEN® QIAamp® Viral Mini QIAcube® kit (Catalog #52926), Valencia, CA.
m. QIAGEN® QIAcube, Valencia CA.

n. Tetracore Inc., Rockville, MD.
io. Universal Influenza A Matrix MPX 2.0, Tetracore, Inc., Rockville, MD.
p. MicroAmp® Optical 96-Well Reaction Plate, Applied Biosystems®, Carlsbad, CA.
r. MagMAX™ Pathogen RNA/DNA Kit (part no.4462359), Applied Biosystems®, Carlsbad, CA.
s. MagMAX™ Express-96 Magnetic Particle Processor, Applied Biosystems®, Carlsbad, CA.
u. Xeno™ RNA Control, Applied Biosystems®, Carlsbad, CA.
v. MagMax 96 Viral RNA Isolation kit (Catalog# AMB1836-5), Applied Biosystems™, Carlsbad, CA.
w. QIAGEN® BioSprint 96, Valencia, CA.
x. KingFisher96 program AM_1836_DW50_v2, KingFisher H 96 magnetic particle processor, Thermo Fisher Scientific Inc., Waltham, MA.
y. Corning, Corning, NY.
z. National Veterinary Services Laboratory, Ames, IA.
aa. Worthington Biochemical, Lakewood, NJ.
ab. Millipore, Billerica, MA.
ac. Gibco®, Grand Island, NY.
ad. Gibco®, Grand Island, NY.
ae. MP Biomedicals, Santa Ana, CA.
af. Acrodisc®, Pall Corporation, Port Washington, NY
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APPENDIX - INFLUENZA A VIRUS DIAGNOSTIC PROCEDURES

Protocols for real-time, reverse transcription polymerase chain reaction (rRT-PCR)

IAV rRT-PCR procedure 1  OF specimens (200μl) were centrifuged (10,000 x g for 30 sec) prior to extraction. Supernatant (140μl) was extracted and purified according to manufacturer’s recommendations and processed according to manufacturer’s recommendations. The inhibition control (IC) was incorporated into the extraction process and used as an extraction and PCR inhibition control for each sample. Final extracted sample elution volume was 60μl. Extracted samples were stored at 4°C and tested within a day.

The rRT-PCR assay was performed using commercial reagents and a dry master mix prepared according to manufacturer’s recommendations. Each reaction (20μl of rehydrated master mix and 5μl of sample) was loaded into one well of a 96-well plate. The reactions were run with the following cycling conditions: 50°C for 30 minutes (Stage 1), then 95°C for 2 minutes (Stage 2), followed by 45 cycles of 95°C for 15 sec, 52°C for 15 sec, and 60°C for 33 sec (Stage 3). The thermocycler was run in “standard” mode and fluorescence data was collected during the 60°C step in the FAM™ and CY5 channels. The baselines were set automatically and the thresholds were set manually for each channel. A sample was considered positive for IAV if it yielded a FAM Ct of < 37. A sample was re-extracted and re-tested if (1) it yielded a FAM Ct of >37 or (2) it yielded a negative CY5 Ct indicative of PCR inhibitors in the reaction.

IAV rRT-PCR procedure 2  OF lysate was prepared by adding OF specimens (300μl) to lysis binding solution, and pre-clarified (16,000 x g for 2 min). Nucleic acid from OF samples was extracted and purified using the entire lysate according to manufacturer’s recommendations using a commercial kit and processor. Final extracted sample elution volume was 90μl. Extracted samples were stored at 4°C and tested within a day.

The IAV rRT-PCR was performed using commercial reagents and an internal control was used as an extraction and PCR inhibition control for each sample. The rRT-PCR reaction
was run with the following cycling conditions: 48°C for 10 minutes (Stage 1), then 95°C for 10 minutes (Stage 2), followed by 40 cycles of 95°C for 15 sec, and 60°C for 45 sec (Stage 3). The thermocycler was run in “standard” mode and fluorescence data was collected during the 60°C annealing/extension stage. Analysis was performed using the control-based threshold setting, with thresholds for SIV-RNA set at 5% of the positive control dRN at cycle 40. A sample was considered positive for IAV if it yielded a Ct of < 38.

**rRT-PCR procedure 3**  OF specimens (280μl) were centrifuged (10,000 x g for 30 sec) prior to extraction. Supernatant (175 μl) was extracted and purified with exceptions, according to manufacturer’s recommendations and processed according to manufacturer’s recommendations. Exceptions to the extraction procedure were as follows: preparation of the lysis binding solution (237μl of lysis/binding solution concentrate (without isopropanol), and 3μl of carrier RNA per reaction); the lysis step (sample supernatant added to the lysis/binding solution with 4μl of IC, then mixed and clarified by centrifugation (14,000 x g for 3 min)); the preparation of the lysis/binding plate (85μl of bead mix, 65μl of 100% isopropanol, and 115μl of lysate added in order to each well). The remaining reagents were prepared according to manufacturer’s recommendations. Final extracted sample elution volume was 75μl. Extracted samples were stored at 4°C and tested within a day.

The rRT-PCR assay was performed using commercial reagents and a dry master mix prepared according to manufacturer’s recommendations. Each reaction (20ul of rehydrated master mix and 5ul of sample) was loaded into one well of a 96-well plate. The reactions were run with the following cycling conditions: 50°C for 30 minutes (Stage 1), then 95°C for 2 minutes (Stage 2), followed by 45 cycles of 95°C for 15 sec, 52°C for 15 sec, and 60°C for 33 sec (Stage 3). The thermocycler was run in “standard” mode and fluorescence data was collected during the 60°C step in the FAM™ and CY5 channels. The baselines were set automatically and the thresholds were set manually for each channel. A sample was considered positive for IAV if it yielded a FAM Ct of < 37. A sample was re-extracted and re-tested if (1) it yielded a FAM Ct of >37 or (2) it yielded a negative CY5 Ct indicative of PCR inhibitors in the reaction.
Protocols for virus isolation

IAV virus isolation procedure 1    The following protocol is fully described elsewhere (Jenkins-Moore, 2010). Briefly, confluent monolayers of Madin-Darby Canine Kidney (MDCK) cells were prepared in 25 cm² flasks. Cell culture media was removed and monolayers were washed 3 times with minimal essential medium (MEM, pH 7.2) containing Earl’s salts, 3X antibiotic solution (25 IU/ml penicillin/ 75 μg/ml streptomycin/ 75 μg/ml gentamicin/ 3 μg/ml amphotericin B), and 5 μg/ml TPCK-treated trypsin (MEM/3X/Tr). 2 ml of OF sample was placed in each flask and incubated at 37°C for 60 min, after which the inoculum was removed. Cell monolayers were rinsed 3 times with MEM/3X/Tr, then replaced with 8-10ml MEM/3X/Tr and incubated for 5-7 days. Cell cultures were evaluated for the appearance of cytopathic effect (CPE) daily. If CPE was present, cell culture was tested by indirect fluorescent antibody detection (IFA) or rRT-PCR. Cells with no CPE were subjected to one freeze-thaw cycle (-80°C and 37°C) and re-inoculated onto fresh MDCK cells. Contaminated cell culture fluids were filtered (0.45 µm) and re-inoculated onto fresh MDCK cells. Samples were considered IAV negative if CPE and IFA were negative after the second passage on cell culture.

IAV virus isolation procedure 2    This protocol is described in detail elsewhere (Leslie-Steen, 2007). Briefly, confluent monolayers of MDCK cells were prepared in 25 cm² flasks. Cell culture media was removed and monolayers washed one time with Hanks balanced salt solution (HBSS). 0.5 ml of OF sample was placed in each flask and incubated at 37°C for 1 hr, after which the inoculum was replaced with minimum essential media (MEM) with Earl’s Salts and L-glutamine, 2 μg/ml sterile filtered porcine pancreas origin trypsin and antibiotics (25 IU penicillin/25 μg streptomycin). Cell cultures were incubated at 37°C with 5% CO₂ and evaluated for the appearance of cytopathic effect (CPE) daily for 5-7 days. CPE-positive cell cultures were confirmed positive by fluorescent antibody (FA). Cells with no CPE were subjected to one freeze-thaw cycle (-80°C and 37°C) and reinoculated on to MDCK cells. Contaminated cell culture fluids were filtered (0.22 µm) and reinoculated on to MDCK cells or the original sample was re-tested. Samples were considered IAV negative if CPE and FA were negative after the second passage on cell culture.
Table 1. IAV rRT-PCR positive samples and mean Ct by dilution* (assays listed in descending order of performance)

<table>
<thead>
<tr>
<th>Assay</th>
<th>Lab</th>
<th>Neg§</th>
<th>Swine oral fluid with H1N1†</th>
<th>Swine oral fluid with H3N2‡</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>$10^{-1}$ $10^{-2}$ $10^{-3}$ $10^{-4}$ $10^{-5}$ $10^{-6}$ $10^{-7}$ $10^{-8}$</td>
<td>$10^{-1}$ $10^{-2}$ $10^{-3}$ $10^{-4}$ $10^{-5}$ $10^{-6}$ $10^{-7}$ $10^{-8}$</td>
</tr>
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<td>10 10 10 10 10 10 6 1 0</td>
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<tr>
<td></td>
<td></td>
<td>36.3</td>
<td>16.6 19.9 23.2 26.5 29.9 33.3 35.4 36.1</td>
<td>19.7 22.9 26.1 29.5 32.6 35.7 36.2 -</td>
</tr>
<tr>
<td>PCR 2</td>
<td>A</td>
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<td>10 10 10 10 10 10 5 2 1</td>
<td>10 10 10 10 10 10 5 2 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>39.2</td>
<td>19.9 23.5 27.0 30.4 33.8 37.3 39.6 39.5</td>
<td>19.7 22.9 26.1 29.4 32.6 35.7 36.2 -</td>
</tr>
<tr>
<td>PCR 3</td>
<td>G</td>
<td>2</td>
<td>10 10 10 10 10 10 10 4 1</td>
<td>10 10 10 10 10 10 3 1 0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>37.7</td>
<td>18.7 22.1 25.4 28.6 32.0 35.6 36.9 38.5</td>
<td>21.7 25.2 28.4 31.6 34.7 36.0 36.7 -</td>
</tr>
<tr>
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<td>33.6 22.2 25.4 28.5 32.2 34.7 33.6 38.5 -</td>
</tr>
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* Results based on a sample set composed of 20 negative controls and 10 samples at each dilution ($10^{-1}$ to $10^{-8}$)
†Concentration of undiluted A/Swine/Ohio/511445/2007 γ H1N1 virus estimated at 1x10$^{-7.5}$/ml TCID$_{50}$
‡Concentration of undiluted A/Swine/Illinois/02907/2009 Cluster IV H3N2 virus estimated at 1x10$^{-6.6}$/ml TCID$_{50}$
§ Detection represents false positive results
Table 2. IAV virus isolation positive samples by dilution* (assays listed in descending order of performance)

<table>
<thead>
<tr>
<th>Assay</th>
<th>Lab§</th>
<th>Neg¦</th>
<th>Swine oral fluid with H1N1†</th>
<th>Swine oral fluid with H3N2‡</th>
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<td></td>
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<td>10⁻²</td>
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</tr>
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<td>0</td>
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<td>10</td>
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<td>VI 7</td>
<td>C</td>
<td>0</td>
<td>9</td>
<td>4</td>
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</tbody>
</table>

* Results based on a sample set composed of 20 negative controls and 10 samples at each dilution (10⁻¹ to 10⁻⁸)
† Concentration of undiluted A/Swine/Ohio/511445/2007 γ H1N1 virus estimated at 1x10⁻⁷.⁵/ml TCID₅₀
‡ Concentration of undiluted A/Swine/Illinois/02907/2009 Cluster IV H3N2 virus estimated at 1x10⁻⁶.⁶/ml TCID₅₀
§ Not all of the 8 laboratories participating in the study conducted virus isolation
¦ Detection represents false positive results
Figure 1. Summary of Ct-values based on influenza A virus rRT-PCR positive swine oral fluid samples by serial dilution and virus subtype (H1N1 or H3N2).
Figure 2. Probability of detecting influenza A virus in swine oral fluid by rRT-PCR based on combined H1N1 and H3N2 testing results. *Differences among superscripts reflect significant differences in assay performance ($p < 0.05$) among the 13 assays. †Duplicate line patterns of the same color indicate identical probability curves.
Figure 3. Probability of detecting influenza A virus in swine oral fluid by virus isolation based on combined H1N1 and H3N2 testing results. *Differences among superscripts reflect significant differences in assay performance ($p < 0.05$) among the 7 assays.
CHAPTER 3. PROBABILITY OF THE DETECTION OF INFLUENZA A VIRUS SUBTYPES H1N1 AND H3N2 IN INDIVIDUAL NASAL SWAB AND PEN-BASED ORAL FLUID SPECIMENS FROM PIGS OVER TIME


A paper submitted to Veterinary Microbiology

ABSTRACT

The probability of detecting influenza A virus (IAV) by virus isolation (VI), point-of-care (POC) antigen detection, and real-time reverse-transcription polymerase chain reaction (rRT-PCR) was estimated for pen-based oral fluid (OF) and individual pig nasal swab (NS) specimens. Piglets (n = 82) were isolated for 30 days and confirmed negative for porcine reproductive and respiratory syndrome virus, Mycoplasma hyopneumoniae, and IAV infections. A subset (n = 28) was vaccinated on day post inoculation (DPI) -42 and -21 with a commercial multivalent vaccine. On DPI 0, pigs were intratracheally inoculated with contemporary isolates of H1N1 (n = 35) or H3N2 (n = 35) or served as negative controls (n = 12). OF (n = 370) was collected DPI 0-16 and NS (n = 924) DPI 0-6, 8, 10,12,14,16. The association between IAV detection and variables of interest (specimen, virus subtype, assay, vaccination status, and DPI) was analyzed by mixed-effect repeated measures logistic regression and the results used to calculate the probability ($\hat{p}$) of detecting IAV in OF and NS over DPI by assay. Vaccination (p-value <0.0001), DPI (p-value <0.0001), and specimen-assay interaction (p-value <0.0001) were significant to IAV detection, but virus subtype was not (p-value = 0.89). Vaccination and/or increasing DPI reduced $\hat{p}$ for all assays. VI was more successful using NS than OF, but both VI and POC were generally unsuccessful after DPI 6. Overall, rRT-PCR on OF specimens provided the highest $\hat{p}$ for the most DPIs, yet significantly different results were observed between the two laboratories independently performing rRT-PCR testing.
INTRODUCTION

Once considered a seasonal infection with rapid onset and recovery (Van Reeth et al., 2012), influenza A virus (IAV) in contemporary commercial swine populations is a chronic, endemic disease with significant herd-level economic effects and broad public health implications. As a result of public health concerns, IAV monitoring in swine has been initiated in some areas, but primarily as a passive system based on testing convenience samples selected from routine case submissions to veterinary diagnostic laboratories (USDA, 2011).

If it is desirable to routinely surveil swine populations, either to guide herd-level interventions or to document the emergence of IAV variants, simple, inexpensive methods of sampling and reliable methods of testing are needed. Nasal swabs (NS), the traditional ante mortem specimen for IAV detection, do not meet these sampling criteria because it is necessary to select, restrain, and swab individual pigs. Alternatively, oral fluids (OF), a specimen new to swine diagnostics but well-characterized in human diagnostics (Prickett and Zimmerman, 2010), is easy to collect because pigs naturally investigate their environment by chewing (Kittawornrat and Zimmerman, 2011). To evaluate the potential of IAV surveillance based on OF sampling, the probability of detecting IAV in OF and NS specimens collected from vaccinated or unvaccinated pigs was compared for 16 days following inoculation with contemporary H1N1 or H3N2 isolates.

MATERIALS AND METHODS

Experimental design
IAV vaccinated and unvaccinated pigs were inoculated with subtypes H1N1 or H3N2. Pen-based oral fluid samples were collected day post inoculation (DPI) 0-16 and individual pig nasal swab samples were collected DPI 0-6, 8, 10, 12, 14, and 16. Specimens were tested for IAV by virus isolation (VI), a "point of care" (POC) rapid antigen test (VetScan™, Abaxis Inc., Union City, CA), and two real-time reverse-transcription polymerase chain reaction
(rRT-PCR) assays. Statistical analyses were performed to define the effect of assay, specimen, virus subtype, vaccination status, DPI, and their interactions on virus detection.

**Influenza A viruses**

Isolate A/Swine/Ohio/511445/2007 γ H1N1 virus (provided by Dr. Amy Vincent, USDA National Animal Disease Center, Ames, IA) was recovered from an influenza outbreak at a county fair in Ohio. A/Swine/Illinois/02907/2009 Cluster IV H3N2 virus (provided by Dr. Marie Gramer, University of Minnesota, St. Paul, MN) was isolated from clinically-affected finishing pigs in Illinois. Both viruses were propagated in Dr. Vincent's laboratory on Madin-Darby Canine Kidney (MDCK) cells to achieve virus concentrations of approximately $1 \times 10^{6.5}$ median tissue culture infectious dose (TCID$_{50}$) per ml.

**Animals and animal care**

The study was conducted in compliance with the Iowa State University (ISU) Institutional Animal Care Use Committee (#11-09-6834-S) and the Institutional Biosafety Committee (09-I-0028-A) guidelines. ISU is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

IAV-negative piglets (n = 82) were obtained from one 600 sow farm at ~21 days of age and an average weight of 6.26 kg (13.8 pounds). Ten days prior to receipt of study piglets, sows in breeding and gestation phases and non-study piglets on sows were bled based on 90% confidence of detecting 10% prevalence. Sow and non-study piglet serum were tested for antibodies against IAV, porcine reproductive and respiratory syndrome virus (PRRSV), and *Mycoplasma hyopneumoniae* and confirmed negative. Likewise, serum samples were tested by PRRSV rRT-PCR using pooled serum samples (5 samples per pool) to confirm freedom from acute PRRSV infection.

The source farm routinely vaccinated piglets against porcine circovirus type 2 (PCV2) at 5 and 21 days of age using a commercial PCV2 vaccine (Circumvent®PCV Intervet/Shering-Plough Animal Health, Millsboro, DE). On DPI -28, i.e., at ~36 days of age, all piglets were
revaccinated against PCV2 (Suvaxyn® PCV2 One-Dose, Fort Dodge Animal Health, New York NY) according to label instructions.

Pigs were fed age-appropriate, commercially-formulated diets throughout the study. Post weaning, piglets were fed a commercially-formulated, pelleted, whey-based, starter diet medicated with chlortetracycline (35g/ton) and tiamulin (400g/ton, Denagard®, Novartis Animal Health US, Inc., Greensboro, NC ; United Farmers Coop, Lesueur, MN). At approximately 49 days of age and for the remainder of the study, they were fed commercial, non-medicated corn:soy meal diets (United Farmers Coop, Lesueur, MN; Heartland Coop, Des Moines, IA).

**Study timeline and treatments**

On DPI -43, the 82 ~21-day-old piglets were moved from the sow farm into one room of a disinfected isolation facility. Serum samples were collected on DPI -42, -21, -7, and 0 and tested for antibodies against IAV, PRRSV, and *M. hyopneumoniae* to document the continued negative status of the group. On DPI -42, each animal was visually inspected and ear tagged. Pigs were randomized to one of six 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. Pigs were housed as one group in the isolation facility; therefore, ear tag color was used to differentiate pigs in IAV vaccinated (n = 28) and nonvaccinated (n = 54) treatment groups. Vaccinated pigs were intramuscularly administered a trivalent commercial IAV vaccine (Flu-Sure® XP, Pfizer Animal Health, Madison, NJ) [A/Swine/Iowa/110600/00 γ (H1N1), A/Swine/North Carolina/031/05 δ (H1N1), and A/Swine/Missouri/069/05(H3N2)] according to label instructions on DPI -42 and DPI -21. The γ H1N1 and Cluster 4 H3N2 components of the vaccine were 95.4% and 98.4% homologous to the hemagglutinin (HA) amino acid sequences of the H1N1 and H3N2 viruses used to inoculate pigs.

On DPI -10, the animals were moved to the ISU Livestock Infectious Disease Isolation Facility, placed 3 or 4 pigs per pen, and allowed to acclimate for ten days. To accommodate the number of pigs and pens, the H1N1 and H3N2 inoculant groups (35 pigs each) were each
housed in two rooms: one room of 5 pens and one room of 6 pens. Vaccinates were housed with their inoculant group, but were penned separately from non-vaccinates. The control group was housed in four pens configured in a 2 x 2 arrangement in one room. Pigs were observed daily throughout the experiment. Manual contact was used to stimulate movement or assess lethargy, if animals were reluctant to rise. Individual animal weights were obtained at DPI -42 and DPI 0 using portable electronic scales (Siltec® WS500 Electronic Weighing Scale Bradford, MA) for the purpose of assessing general health and group uniformity.

On DPI 0, animals in inoculant groups H1N1 and H3N2 were intratracheally administered 2 ml of a solution containing $1 \times 10^{6.5}$ TCID$_{50}$ per ml of either A/Swine/Ohio/511445/2007 γ H1N1 or A/Swine/Illinois/02907/2009 cluster IV H3N2. A portion of each viral inoculum was stored (-80°C) for back-titration.

Serum samples
Serum samples were collected on DPI -42, -21, -7, 0, 7. Blood samples were drawn from the jugular vein or cranial vena cava using a single-use blood collection system (Becton Dickson, Franklin Lakes, NJ) and serum separation tubes (Kendall, Mansfield, MA). Samples were centrifuged at 1800 x g for 10 min at 4°C, after which sera were aliquoted into 5 ml tubes (BD Falcon™, Franklin Lakes, NJ) and stored at -20°C until tested.

Oral fluid samples
Oral fluid samples were collected daily DPI -5 to 16. To familiarize pigs with the rope used for collecting oral fluid samples, 45 cm (18") of 1.3 cm (1/2") 3-strand, undyed, unbleached 100% cotton rope (Web Rigging Supply, Inc., Lake Barrington, IL) was knotted and placed on the floor of each pen for 20 minutes in the morning and afternoon on DPI -6. Thereafter, collections were performed by suspending one rope in each pen for 30 minutes each morning. Oral fluids were absorbed as the pigs played with (chewed on) the rope. To recover the specimen, the bottom 15.2 cm (6") of the rope (wet portion) was inserted into a one gallon re-sealable plastic bag and severed from the dry portion of the rope. The entire bag with the wet rope inside was passed through a wringer (Dyna-Jet Products, Overland Park, KS).
Park, KS), causing the fluid to pool in the bottom of the bag. A corner of the bag was cut and the contents drained into a 50 ml conical-bottom 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. To avoid contamination between treatment groups, three people collected and processed ropes, i.e., one person for each treatment group (negative control, H1N1, H3N2). Ropes were processed prior to leaving the treatment group housing area. Immediately following collection, samples were refrigerated (4°C), centrifuged at 13,000 x g for 10 min at 4°C, aliquoted into 5 ml snap cap tubes (BD Falcon, Fisher Scientific) and stored at -80°C.

**Nasal swab samples**

Nasal swabs were collected on DPI 0-6, 8, 10, 12, 14, and 16 from all inoculated pigs and DPI 0-4, 8, 12 and 16 from negative control pigs. To collect samples, a swab (Copan™ minitip flocked swab, Fisher Scientific No. 501CS01) was inserted ~8 cm into one nasal passage of the pig, rotated, and removed. The naris sampled was alternated each day, i.e., all left nares one day and all right nares the next. Following sampling, the swab was broken off into 3 ml of viral transport medium (BD Universal Transport Media with modified Hank’s balanced salt solution, Fisher Scientific No. 220220). In the laboratory, the tubes containing swabs were vortexed, the media aliquoted into two ml cryovials (Corning®), and the samples were stored at -80°C.

**Testing procedures**

*Pre-inoculation samples* To verify the negative status of the group prior to inoculation with IAV, serum samples collected on DPI -42, -21, -7, 0, 7 were tested for evidence of infection with PRRSV, *Mycoplasma hyopneumoniae*, and IAV.

Serum samples were tested for IAV antibody using a blocking ELISA (IDEXX AI MultiS-Screen Ab Test, IDEXX Laboratories, Inc., Westbrook, ME) using a cut-off of S/N = 0.67 (Ciacci-Zanella et al., 2010). The assay was conducted according to the manufacturer's instructions and the laboratory's standard operating procedure (Boesenberg, 2012).
Consistent with the other ELISAs, quality control included statistical process control (SPC) charting of in-house negative and positive controls (Northwest Analytic SPC, Portland, OR). Data management and calculations were performed using the software provided by the manufacturer. Results were reported as S/N (sample/kit negative control) ratios.

Indirect antibody ELISAs licensed for testing swine serum (IDEXX PRRS X3 Ab Test, IDEXX Laboratories, Inc., Westbrook, ME; IDEXX M hyo Ab Test, IDEXX Laboratories, Inc., Westbrook, ME) were performed according to the manufacturer’s instruction and analyzed using the manufacturer's software (xChek®, IDEXX Laboratories, Inc., Westbrook M.E). Negative and positive controls provided by the manufacturer were run on all plates and the performance of each plate was validated using the manufacturer's software. As an additional quality control step, in-house negative and positive controls were run on each M. hyopneumoniae ELISA plate and four in-house controls (negative, low, intermediate, and high positives) were run on each PRRSV ELISA plate. In-house and kit control results were analyzed by SPC charting (Northwest Analytic SPC).

Pre-inoculation serum samples were pooled in groups of 4 or 5 and tested by PRRSV rRT-PCR. Nucleic acid was extracted (MagMax™ 96 Viral RNA Isolation Kit, Applied Biosystems®, Foster City, CA) and purified (Kingfisher® 96, Thermo Electron, West Palm Beach, FL) according to manufacturer’s recommendations. PRRSV rRT-PCR was performed using NA and EU PRRSV Reagents (TaqMan® NA and EU PRRSV Reagents, Applied Biosystems®, Foster City, CA) with master mix made following the manufacturer’s recommendations. An internal RNA control (Xeno®, Applied Biosystems®, Foster City, CA) was included at the master mix step. The reactions were run (Applied Biosystems® 7500 Fast Real-Time PCR System, Applied Biosystems®, Foster City, CA) with cycling conditions as follows: 45°C for 10 minutes, then 95°C for 10 minutes, followed by 40 cycles of 97°C for 2 sec, and 60°C for 40 sec. The instrument was run in “fast” mode and florescence data was collected during the 60°C annealing/extension stage. Analysis was performed using the auto baseline setting, with thresholds for NA and EU PRRSV set at 0.2 and 0.05, respectively. A sample was considered positive for the respective PRRSV
genotype if it yielded a cycle threshold (Ct) of \( \leq 37 \).

*Post-inoculation IAV samples*  Oral fluid and nasal swabs collected between DPI 0 and 16 were tested for IAV by rRT-PCR (Laboratories A and B), VI, and POC assays. Testing was blinded by complete randomization of samples prior to submission.

*Laboratory A rRT-PCR protocols for matrix and hemagglutinin genes*  OF or NS specimens (180μl) were centrifuged (14,000 x g for 30 sec) prior to extraction and then 140μl 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) on the QIAGEN® QIAcube® processor. The inhibition control (IC) was used as an extraction and PCR inhibition control for each sample. The rRT-PCR assay was performed using commercial reagents (Universal Influenza A Matrix MPX 2.0, Tetracore, Inc., Rockville, MD) 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) with the following cycling conditions: 50°C for 30 minutes (reverse transcription), then 95°C for 2 minutes (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 IAV matrix target if it yielded a Ct of < 37. Matrix-positive samples were tested by rRT-PCR for H1 and H3 genes. The H1 assay was performed using a protocol described in detail elsewhere (WHO, 2009), with primers and probes obtained from Biosearch Technologies (Novato, CA), and the positive control from Integrated DNA Technologies, Inc. (Coralville, IA). The H3 assay was performed using a protocol described elsewhere (Richt et al., 2004), with primers and probes obtained from Integrated DNA Technologies, Inc. and a positive control consisting of a dilution of extracted H3 RNA from a previous study.
Laboratory B rRT-PCR protocols for matrix and hemagglutinin genes

OF (300μl) or NS (50μl) samples were assayed using a commercial kit performed as instructed by the manufacturer (Swine Influenza Virus RNA Test Kit Document part number 4444272 Rev B, Applied Biosystems®). Briefly, nucleic acid from OF and NS samples was extracted and purified using MagMAX™ Pathogen RNA/DNA Kit (part no.4462359). IAV rRT-PCR was performed using 2x Multiplex RT-PCR Enzyme Mix, SIV Primer probe mix, Xeno® RNA control (Applied Biosystems®, Foster City, CA, part no. 4415200) and SIV-Xeno® RNA Control Mix. The reactions were run (Applied Biosystems® 7500 Fast Real-Time PCR System, Applied Biosystems®, Foster City, CA) with the following cycling conditions: 48°C for 10 minutes (reverse transcription), then 95°C for 10 minutes (RT inactivation/initial denaturation), followed by 40 cycles of 95°C for 15 sec, and 60°C for 45 sec (amplification). The thermocycler was run in “standard” mode and fluorescence data was collected during the 60°C annealing/extension stage. Analysis was performed using the control-based threshold setting, with thresholds for SIV-RNA set at 5% of the positive control dRN at cycle 40. A sample was considered positive for IAV if it yielded a Ct of < 38. Matrix-positive samples were tested by rRT-PCR for H1 and H3 genes using non-commercial in-house reagents with one primer/probe mix amplifying both H1 and H3 RNA in a single multiplex reaction. The H1 probe was labeled with a VIC® fluorophore (Applied Biosystems®) and the H3 was labeled with FAM™ (Applied Biosystems®). Using the previously extracted RNA, rRT-PCR was performed using 2x Multiplex RT-PCR buffer (Applied Biosystems®), enzyme mix (Applied Biosystems®), and H1H3 primer probe mix (Applied Biosystems®).

Virus Isolation

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, St. Louis, MO), streptomycin (300 μg/ml; Sigma-Aldrich, St. Louis, MO), gentamicin (150 μg/ml; Sigma-Aldrich, St. Louis, MO) and amphotericin B (0.75 μg/ml; Gibco, Grand Island, NY)], and TPCK-treated trypsin (2 μg/ml; Sigma-Aldrich. St. Louis, MO). Prior to inoculation on to MDCK cells, 0.35 μl of antibiotic-antimycotic
solution was added to each 1 ml oral fluid and nasal swab sample, after which samples were held at room temperature for 1hr. Each OF and NS sample was divided among 3 wells, i.e., ~0.4 ml per well, and then incubated at 37°C with 5% CO₂ for 2 hrs, after which the inoculum was removed. Cell monolayers were rinsed 3 times with the IAV wash solution, and then 0.4ml IAV post-inoculation media composed of MEM with Earle's salts, 3x antibiotic-antimycotic solution, and TPCK-treated trypsin (1.5μg/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 by for IAV by rRT-PCR. Cells with no CPE were subjected to two 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 on to fresh confluent MDCK cells in 3 wells. Samples were considered IAV 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 point-of-care (POC) antigen test The POC assay (VetScan®, Abaxis Inc.) evaluated was a rapid, immunochromatographic avian IAV antigen assay USDA licensed for chicken, turkey, and duck tracheal, oropharyngeal, and cloacal swab specimens. Oral fluid and nasal swab samples were tested according to the manufacturer’s instructions for avian samples. In brief, 100μl of sample was added to the devise and it was sealed closed. Nasal swab results were read at 15 minutes and oral fluid results were read at both 15 and 30 minutes. Results were interpreted as described by the manufacturer.

Data analysis
A mixed-effect repeated measures logistic regression model (Proc GLIMMIX, SAS® Version 9.3, SAS® Institute, Inc., Cary, NC) was used to analyze the association between the detection of IAV and the variables of interest: sample specimen (OF or NS), virus subtype (H1N1 or H3N2), assay (VI, POC, rRT-PCR), vaccination status (yes/no), and DPI. Within the limitations of the dataset, interactions of specimen with other variables of interest were
tested for significance. Random effects included sample and pen. Fixed effects were considered significant at $\alpha = 0.05$. The probability of detecting IAV in OF and NS over time post inoculation was estimated for rRT-PCR, VI, and POC tests from the mixed-effect repeated measures logistic regression model. Likewise, the model was used to calculate the probability of detection for both rRT-PCR laboratories over time. A nonlinear mixed model (Proc NLMIXED, SAS® Version 9.3) was used to estimate mean rRT-PCR Ct values for each laboratory as a function of vaccination status and specimen over DPI. Ct cutoff values for each laboratory served as boundary constraints.

RESULTS

General observations All serum samples collected on DPI -42, -21, -7, and 0 were ELISA negative for *M. hyopneumoniae*, PRRSV and IAV antibodies, as well as negative for PRRSV by rRT-PCR. At the time of inoculation (DPI 0), all animals appeared clinically normal and no statistically significant difference in pig weight by treatment group was detected. Back titration of the H1N1 and H3N2 inocula yielded estimates of $1 \times 10^{6.5}$ and $1 \times 10^{5.7}$ TCID$_{50}$/ml for A/Swine/Ohio/511445/2007 γ H1N1 and A/Swine/Illinois/02907/2009 Cluster IV H3N2, respectively. Pigs were reluctant to move on DPI 1, but not on DPI 2 or later. One pig was treated for polyarthritis with ceftiofur sodium (Excede® for swine, Pfizer Animal Health, 5 mg/kg) on DPI 4, but no animals required treatment for respiratory disease and no animals were removed from the study. A total of 370 OF samples and 924 NS samples were collected for testing (Table 1). Results are summarized by assay and treatment group in Table 2.

Receiver operator characteristic (ROC) analyses (MedCalc® Version 12.3.0.0, MedCalc Software, Mariakerke, Belgium) of the 2 OF POC protocols (15 min vs. 30 min incubation) for DPIs 1-6 showed that longer incubation resulted in improved overall diagnostic sensitivity, i.e., 41.6% (47/113) with 15 min incubation versus 51.3% (58/113) with 30 min incubation (p-value = 0.0015). Prolonged incubation did not affect the specificity (100%, 113/113) of the POC for OF. Because the 30 min incubation POC assay was shown to be more diagnostically sensitive, the 30 min results were used in the statistical model.
An assessment of the association between the number of NS positive pigs within the pen and the detection of IAV in pen-based OF is given in Table 3 by assay. For all assays, the likelihood of IAV detection in OF increased as the number of NS positive pigs in the pen increased. Most notably, the likelihood of an rRT-PCR result was $>70\%$ with one NS rRT-PCR positive pig in the pen, but the probability was $>30\%$ even when zero NS positive pigs were detected.

Analysis of the data in a mixed-effect logistic regression model identified the variables significant to IAV detection as: vaccination status ($p$-value $<0.0001$), DPI ($p$-value $<0.0001$), and specimen• assay ($p$-value $<0.0001$). Virus subtype was not significant to IAV detection ($p$-value $= 0.89$); therefore, these data were subsumed into a single variable for subsequent analyses. The probability of IAV detection ($\rho$) over time by specimen ($\rho_{OF}$, $\rho_{NS}$) was calculated using Equations 1 and 2, with results for unvaccinated and vaccinated pigs shown in Figures 1 and 2, respectively.

$$\lambda_{\text{specimen type}} = \text{Logit}(P(X)) = \alpha + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3$$  
Equation 1

$\alpha = \text{intercept}$

$\beta_1 = \text{regression coefficient for vaccination status (referent: vaccinated)}$

$\beta_2 = \text{regression coefficient for DPI 1-16 (referent: DPI = 1)}$

$\beta_3 = \text{regression coefficient for assay (referent: POC)}$

$$\text{Probability}_{\text{specimen type}} = (\rho_{OF,NS}) = \frac{e^{(\lambda)}}{1 + e^{(\lambda)}}$$  
Equation 2

The calculated probability ($\hat{\rho}$) differed significantly between unvaccinated and vaccinated pigs (Table 4). In unvaccinated pigs, rRT-PCR $\hat{\rho}_{OF}$ and $\hat{\rho}_{NS}$ were equivalent through DPI 6, then higher in OF through DPI 16. VI was more successful using NS than OF, but VI $\hat{\rho}$ was significantly lower than rRT-PCR $\hat{\rho}$. The POC assay $\hat{\rho}$ was greater in pen-based OF (30 min incubation) from DPI 1-6 than individual NS in unvaccinated groups. After DPI 6, both VI and POC were generally unsuccessful in unvaccinated animals. Vaccination significantly reduced the $\hat{\rho}$ for all assays. For example, the rRT-PCR $\hat{\rho}$ on DPI 1 in vaccinated pigs was
70% for pen-based OF and 11% for NS versus 98% and 88%, respectively for samples from unvaccinated pigs. The highest $\hat{p}$ of isolating virus from OF was ≤12% at any time and ≤46% for individual NS. After DPI 6, $\hat{p}$ approached zero in all assay-specimen combinations except for OF specimens tested by rRT-PCR.

**IAV rRT-PCR performance**  In unvaccinated animals, significant differences between laboratories were observed after DPI 7 (Figure 3a,b), whereas differences were observed at DPI 1 and after DPI 6 in vaccinated animals, (Figure 3a,b). As given in Table 5, within laboratory estimated mean Ct values were essentially equivalent between OF and NS from DPI 0-5 in unvaccinated pigs, after which estimated mean Ct values were lower, i.e., virus RNA concentrations were higher, in OF than NS through DPI 16. Similar observations were made in vaccinated pigs, with sporadic detection in OF after DPI 9.

Results of hemagglutinin subtyping (H1 or H3) on rRT-PCR IAV matrix gene positive samples are reported in Table 6. In both OF and NS samples, correctly identifying subtype ("matching") was significantly related to DPI (p-value < 0.0001) and laboratory (p-value < 0.0001). That is, matching was more successful in early infection and Laboratory B was significantly more likely than Laboratory A to match matrix positive samples to subtype from either OF or NS. Specifically, Laboratory A reported 129 of 605 (21%) of matrix-positive samples as "undetermined" versus 5 of 554 (1%) from Laboratory B. Of the 129 samples with an undetermined subtype from Laboratory A, 33 (26%) were from vaccinated pens. Two of the 5 OF samples (40%) classified as undetermined from Laboratory B were from vaccinated pens.

**DISCUSSION**

The detection of IAV in pen-based oral fluid samples and individual nasal swabs was compared for the first 16 days after intratracheal inoculation using three diagnostic assays (VI, POC, rRT-PCR). The timeline allowed for comparison of the onset, magnitude, and duration of virus shedding for two IAV subtypes in vaccinated and unvaccinated pigs. In recognition of the dynamic nature of IAV, the viruses selected for this study represented
contemporary H1N1 (A/Swine/Ohio/511445/2007 γ H1N1) and H3N2 (A/Swine/Illinois/02907/2009 Cluster IV H3N2) isolates (Vincent et al., 2009; WHO, 2011). The viruses shared 95.4% and 98.4% HA amino acid homology to two of the IAV viruses (A/Swine/Iowa/110600/00 γ H1N1 and A/Swine/Missouri/069/05 H3N2) in the inactivated trivalent commercial vaccine. The three assays evaluated were chosen purposefully: (1) virus isolation is the historical standard for IAV diagnosis, but not necessarily diagnostically sensitive (Cheng et al., 2010; Ganzenmueller et al.; 2010); (2) the POC assay is an animal-side test, but its performance has not been established for swine; and (3) rRT-PCR is the contemporary method of IAV diagnosis, but assay performance estimates are lacking.

A mixed-effect logistic regression model examining the association between IAV detection in specimen (OF or NS), virus subtype (H1N1 or H3N2), assay (VI, POC, rRT-PCR), vaccination status (yes/no), and DPI identified vaccination status (p-value <0.0001), DPI (p-value <0.0001), and the interaction of specimen and assay (p-value <0.0001) as significant. The effect of vaccination and DPI is consistent with current knowledge regarding immunity against IAV whereas the specimen-assay interaction addresses the relationship between diagnostic specimens and assay performance.

IAV infection in healthy, naïve animals produces an anti-IAV immune response that results in a rapid reduction in viral replication and shedding (Van Reeth et al., 2012). Acquired immunity produced by IAV infection includes both cell mediated immunity that functions in viral clearance and heterosubtypic IAV immunity (Flynn et al 1998; Nguyen et al., 1999; Takada et al., 2003, Webster and Askonas, 1980), and anti-IAV antibody on respiratory mucosal surfaces that prevents viral attachment and entry into pulmonary epithelial cells (Larsen et al., 2000). In addition to immunity resulting from primary infection, maternal antibody and immune priming produced by vaccination can also affect the course of infection. Maternally derived antibody (MDA) has been shown to reduce clinical signs in IAV-infected piglets (Kitikoon et al., 2006; Loeffen et al., 2003) and affect detection and transmission of IAV in both homologously and heterologously IAV-challenged piglets (Allerson et al., 2012; Kitikoon et al., 2006).
The ability of vaccine to reduce the magnitude and duration IAV shedding has been reported previously in both mammals and birds (Bikour et al., 1996; Bos et al., 2008; Jones et al., 2011; Paillot et al., 2012; Vincent et al., 2010). This is significant because a reduction in IAV shedding reduces the rate of transmission in pig populations (Allerson et al, 2012; Romagosa et al 2011, 2012; Torremorell et al, 2011). For reasons that are unclear, the degree of immunity conferred by vaccination is variable. That is, protection is not entirely explained by the vaccine and challenge virus hemagglutinin amino acid homology (Vincent et al., 2010; Kyriakis et al., 2010; Van Reeth et al., 2004). The γ H1N1 and Cluster 4 H3N2 components of the commercial vaccine used in this study were 95.4% and 98.4% homologous to the HA amino acid sequences of the H1N1 and H3N2 viruses. Although amino acid homology cannot be directly correlated with protection, the effect of vaccination differed between virus subtypes. As shown in Table 2, IAV was isolated from 28 of 114 (24.6%) NS samples collected from H1N1 inoculated, vaccinated pigs (group V_H1) versus 1 of 119 (0.8%) NS samples collected from H3N2 inoculated, vaccinated pigs (group V_H3).

The interaction of specimen and assay underscores the fact that both the assay and the specimen affect the likelihood of detecting IAV. Virus isolation is time consuming and costly, but VI is currently the only option if it is necessary to recover IAV isolates for further use or analysis. In both unvaccinated and vaccinated pigs, the probability of isolating IAV from NS or OF was highest at DPI 4, with the success of VI declining rapidly thereafter. Isolation was significantly less likely in OF than NS, particularly in vaccinated animals. We hypothesize that this reflects the presence of anti-IAV antibody (both IgG and IgA) in OF (Panyasing et al., 2012).

A marked advantage of the POC is rapid turnaround (30 min) for either OF or NS specimens. The POC assay detected IAV in swine in the first five days of infection with a positive predictive value of 100% (no false positives). However, the assay's diagnostic sensitivity for OF specimens for DPIs 1 to 5 was 51.3%, with better performance in unvaccinated pigs (64.6%) than vaccinated pigs (20.6%). Thus, the test was relatively insensitive and markedly affected by the presence of anti-IAV antibodies.
Relative to VI and POC, rRT-PCR was the most likely to detect IAV for the longest time post inoculation (Figures 1, 2), particularly in OF (Table 4). A comparison of rRT-PCR results from the two independent laboratories showed that the pattern of Ct values was affected by DPI, specimen, and vaccination status. Estimated mean OF and NS Ct values were similar within laboratory until DPI 6 (Table 5), after which estimated OF Ct values trended lower (indicating a higher virus concentration). Since IAV replicates in bronchial epithelial cells, the higher and more prolonged detection of viral RNA in OF could be explained by physical expulsion of virus from the lungs via normal pulmonary clearance mechanisms, such as coughing (Levandowski et al., 1985). Vaccination status affected detection, but the relationship remained the same, i.e., OF estimated mean Ct values were usually equal to, or less than, NS estimated mean Ct values.

A direct statistical comparison of Ct values between laboratories was not possible because of differences in threshold and cutoff values, but it was possible to determine that the probability of detection differed between laboratories. Specifically, probability estimates (\(p\)) based on logistic regression analysis revealed that Laboratory A was significantly more likely to report rRT-PCR positive OF and NS specimens than Laboratory B (Figure 3a,b), while Laboratory B was more likely to correctly identify virus subtype in rRT-PCR-positive samples (Table 6). These results are of concern because surveillance requires highly reproducible and repeatable assays; otherwise, results come to be viewed with doubt and skepticism. Resolution of this issue should be a high priority.

Influenza A virus-associated morbidity and mortality is an economically significant problem in commercial swine populations. For producers and veterinarians, the prevention and/or control of IAV is dependent upon the degree of match between herd immunity (passive and acquired) and the virus strain infecting the herd. Maintaining herd protection is complicated by the constant emergence of new antigenic variants as IAV circulates within, and moves between, susceptible host populations. For example, pH1N1, first identified in April 2009 in humans (Garten et al., 2009), is now globally endemic in swine, co-circulating with
established subtypes and creating novel reassortants (Ali et al., 2012; Chen et al., 2012; Starick et al., 2012).

To respond in a timely fashion to this rapidly evolving scenario, swine producers and veterinarians need population-based sampling methods that provide for the continual assessment of IAV. Historically, ante mortem IAV detection has relied on NS specimens. While NS are the best sample for VI (Table 4), the process of collecting NS specimens is both labor intensive and dependent upon fortuitously selecting a pig in the first 7 days of IAV infection, when virus is still present in nasal secretions (van Reeth et al., 2012). As an alternative, pen-based OF are easily collected and the probability of detecting IAV infection by rRT-PCR was actually shown to be higher than individual pig NS specimens in this study and elsewhere (Romagosa et al., 2012). Previously, shown to be an effective diagnostic specimen for a variety of swine pathogens (Detmer et al., 2010; Kittawornrat et al., 2010; Prickett et al., 2008a,b; Ramirez et al., 2012), OF would appear to be the specimen of choice for the surveillance of IAV.

DECLARATION OF CONFLICTING INTERESTS

The authors declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article. Authors R Rauh, W Nelson, C O'Connell, and A Burrell are employed by private industry. The remaining authors declare no conflicting interests with respect to their authorship or the publication of this article.

ACKNOWLEDGMENTS

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World Health Organization. 2009. CDC protocol of real time RT-PCR for swine influenza A(H1N1). (30 April 2009)

Table 1. Description of treatment groups and sampling

<table>
<thead>
<tr>
<th>Group designation</th>
<th>Negative Control</th>
<th>H1N1 Inoculated&lt;sup&gt;a&lt;/sup&gt;</th>
<th>H3N2 Inoculated&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unvaccinated</td>
<td>Unvaccinated</td>
<td>Unvaccinated</td>
</tr>
<tr>
<td>Vaccination&lt;sup&gt;c&lt;/sup&gt;</td>
<td>UV&lt;sub&gt;CTRL&lt;/sub&gt;</td>
<td>UV&lt;sub&gt;H1&lt;/sub&gt;</td>
<td>UV&lt;sub&gt;H3&lt;/sub&gt;</td>
</tr>
<tr>
<td>Vaccinated</td>
<td>V&lt;sub&gt;CTRL&lt;/sub&gt;</td>
<td>V&lt;sub&gt;H1&lt;/sub&gt;</td>
<td>V&lt;sub&gt;H3&lt;/sub&gt;</td>
</tr>
<tr>
<td>Pigs</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Pens</td>
<td>6</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>OF samples&lt;sup&gt;d&lt;/sup&gt; (n = 370)</td>
<td>28</td>
<td>119</td>
<td>114</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>48</td>
<td>47</td>
</tr>
<tr>
<td>NS samples&lt;sup&gt;e&lt;/sup&gt; (n = 924)</td>
<td>96</td>
<td>264</td>
<td>264</td>
</tr>
<tr>
<td></td>
<td>58</td>
<td>121</td>
<td>121</td>
</tr>
</tbody>
</table>

<sup>a</sup> A/Swine/Ohio/511445/2007 γ H1N1 virus

<sup>b</sup> A/Swine/Illinois/02907/2009 Cluster IV H3N2 virus

<sup>c</sup> Vaccinated on DPI -42 and -21 with a trivalent, inactivated influenza vaccine (Flu-Sure® XP, Pfizer Animal Health, Madison, NJ)

<sup>d</sup> Oral fluid samples collected daily DPI 0 to 16

<sup>e</sup> Nasal swabs collected on DPI 0-6, 8, 10, 12, 14, 16 from all inoculated pigs and DPI 0-2, 4, 8, 12 and 16 from negative control pigs
<table>
<thead>
<tr>
<th>Assay</th>
<th>Negative Control</th>
<th>H1N1 Inoculated</th>
<th>H3N2 Inoculated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unvaccinated</td>
<td>Unvaccinated</td>
<td>Vaccinated</td>
<td>Unvaccinated</td>
</tr>
<tr>
<td>POC OF (15 min incubation)</td>
<td>0/23</td>
<td>23/75</td>
<td>22/66</td>
</tr>
<tr>
<td></td>
<td>30.7%</td>
<td>6.7%</td>
<td>33.3%</td>
</tr>
<tr>
<td>POC OF (30 min incubation)</td>
<td>0/23</td>
<td>28/75</td>
<td>24/66</td>
</tr>
<tr>
<td></td>
<td>37.3%</td>
<td>23.3%</td>
<td>36.4%</td>
</tr>
<tr>
<td>POC NS (15 min incubation)</td>
<td>0/84</td>
<td>67/192</td>
<td>83/191</td>
</tr>
<tr>
<td></td>
<td>34.9%</td>
<td>11.4%</td>
<td>43.5%</td>
</tr>
<tr>
<td>Virus isolation oral fluids</td>
<td>0/26</td>
<td>26/114</td>
<td>7/107</td>
</tr>
<tr>
<td></td>
<td>22.3%</td>
<td>2.1%</td>
<td>6.5%</td>
</tr>
<tr>
<td>Virus isolation nasal swabs</td>
<td>1/95</td>
<td>119/257</td>
<td>119/258</td>
</tr>
<tr>
<td></td>
<td>22.3%</td>
<td>24.6%</td>
<td>46.1%</td>
</tr>
<tr>
<td>rRT-PCR OF Laboratory A</td>
<td>2/28</td>
<td>85/119</td>
<td>103/114</td>
</tr>
<tr>
<td></td>
<td>7.1%</td>
<td>58.3%</td>
<td>90.4%</td>
</tr>
<tr>
<td>rRT-PCR OF Laboratory B</td>
<td>1/27</td>
<td>72/115</td>
<td>93/114</td>
</tr>
<tr>
<td></td>
<td>3.7%</td>
<td>43.8%</td>
<td>81.6%</td>
</tr>
<tr>
<td>rRT-PCR NS Laboratory A</td>
<td>5/96</td>
<td>176/264</td>
<td>181/264</td>
</tr>
<tr>
<td></td>
<td>5.2%</td>
<td>49.6%</td>
<td>68.6%</td>
</tr>
<tr>
<td>rRT-PCR NS Laboratory B</td>
<td>1/96</td>
<td>147/264</td>
<td>156/262</td>
</tr>
<tr>
<td></td>
<td>1.0%</td>
<td>37.2%</td>
<td>59.5%</td>
</tr>
</tbody>
</table>

\(^a\) "Point of care" (POC) rapid antigen test (VetScan™, Abaxis Inc.), real-time reverse-transcription polymerase chain reaction (rRT-PCR)

\(^b\) 370 oral fluid (OF) and 924 nasal swab (NS) samples (DPI 0 to 16) were assayed by rRT-PCR and VI. 231 OF and 689 NS samples (DPI 0 to 10) were tested by POC. Differences between the number collected and the number tested represents missing data.

\(^c\) Vaccinated on DPI -42 and -21 with a trivalent, inactivated influenza vaccine (Flu-Sure® XP, Pfizer Animal Health)

\(^d\) A/Swine/Ohio/511445/2007 γ H1N1 virus

\(^e\) A/Swine/Illinois/02907/2009 Cluster IV H3N2 virus
<table>
<thead>
<tr>
<th>Assay used to test OF and NS samples</th>
<th>Number of NS-Positive pigs within a pen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Virus isolation</td>
<td>0/123</td>
</tr>
<tr>
<td></td>
<td>0%</td>
</tr>
<tr>
<td>POCb (OF with 30 min incubation)</td>
<td>7/101</td>
</tr>
<tr>
<td></td>
<td>7%</td>
</tr>
<tr>
<td>rRT-PCRc Laboratory A</td>
<td>23/59</td>
</tr>
<tr>
<td></td>
<td>39%</td>
</tr>
<tr>
<td>rRT-PCRc Laboratory B</td>
<td>28/86</td>
</tr>
<tr>
<td></td>
<td>33%</td>
</tr>
</tbody>
</table>

**Table 3. Detection of IAV in pen-based oral fluid (OF) samples as a function of the number of nasal swab (NS)-positive pigs within a pen**

*a* Table based on days in which both nasal swab and oral fluid samples were collected from pens of IAV-inoculated pigs, i.e., DPI 0-6, 8, 10, 12, 14, 16

*b* "Point of care" (POC) rapid antigen test (VetScan™, Abaxis Inc.) comparison based on samples collected from DPI 1-6, 8, 10.

*c* Real-time reverse-transcription polymerase chain reaction (rRT-PCR)
Table 4. Probability of IAV detection over time in inoculated animals by vaccination status, assay\textsuperscript{a}, and specimen\textsuperscript{b}

<table>
<thead>
<tr>
<th></th>
<th>Unvaccinated, IAV-inoculated</th>
<th>Vaccinated, IAV-inoculated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>rRT-PCR</td>
<td>Virus Isolation</td>
</tr>
<tr>
<td>DPI</td>
<td>OF</td>
<td>NS</td>
</tr>
<tr>
<td>1</td>
<td>0.98</td>
<td>0.88</td>
</tr>
<tr>
<td>2</td>
<td>1.00</td>
<td>0.99</td>
</tr>
<tr>
<td>3</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>4</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>5</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>6</td>
<td>0.99</td>
<td>0.96</td>
</tr>
<tr>
<td>7</td>
<td>0.96</td>
<td>na\textsuperscript{c}</td>
</tr>
<tr>
<td>8</td>
<td>0.90</td>
<td>0.60</td>
</tr>
<tr>
<td>9</td>
<td>0.77</td>
<td>na</td>
</tr>
<tr>
<td>10</td>
<td>0.80</td>
<td>0.20</td>
</tr>
<tr>
<td>11</td>
<td>0.72</td>
<td>na</td>
</tr>
<tr>
<td>12</td>
<td>0.60</td>
<td>0.16</td>
</tr>
<tr>
<td>13</td>
<td>0.61</td>
<td>na</td>
</tr>
<tr>
<td>14</td>
<td>0.53</td>
<td>0.08</td>
</tr>
<tr>
<td>15</td>
<td>0.24</td>
<td>na</td>
</tr>
<tr>
<td>16</td>
<td>0.19</td>
<td>0.03</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Real-time reverse-transcription polymerase chain reaction (rRT-PCR), virus isolation and "Point of care" (POC) rapid antigen test (VetScan\textsuperscript{™}, Abaxis Inc.)

\textsuperscript{b} Oral fluid (OF) and nasal swab (NS) samples

\textsuperscript{c} No sample collected
Table 5. Estimated mean Ct values of rRT-PCR positive samples by vaccination status, laboratory, and specimen\(^a\)

<table>
<thead>
<tr>
<th>DPI</th>
<th>Unvaccinated, IAV-inoculated</th>
<th></th>
<th>Vaccinated, IAV-inoculated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Laboratory A</td>
<td>Laboratory B</td>
<td>Laboratory A</td>
</tr>
<tr>
<td></td>
<td>OF</td>
<td>NS</td>
<td>OF</td>
</tr>
<tr>
<td></td>
<td>x (95% CI)</td>
<td>x (95% CI)</td>
<td>x (95% CI)</td>
</tr>
<tr>
<td>1</td>
<td>27 (26, 28)</td>
<td>30 (29, 31)</td>
<td>32 (31, 33)</td>
</tr>
<tr>
<td>2</td>
<td>18 (17, 19)</td>
<td>21 (20, 22)</td>
<td>23 (22, 23)</td>
</tr>
<tr>
<td>3</td>
<td>16 (15, 17)</td>
<td>19 (18, 20)</td>
<td>21 (20, 22)</td>
</tr>
<tr>
<td>4</td>
<td>16 (15, 17)</td>
<td>19 (18, 20)</td>
<td>20 (20, 21)</td>
</tr>
<tr>
<td>5</td>
<td>17 (16, 18)</td>
<td>20 (20, 21)</td>
<td>22 (21, 23)</td>
</tr>
<tr>
<td>6</td>
<td>22 (21, 23)</td>
<td>25 (24, 26)</td>
<td>27 (26, 28)</td>
</tr>
<tr>
<td>7</td>
<td>27 (25, 28)</td>
<td>na(^b)</td>
<td>32 (30, 33)</td>
</tr>
<tr>
<td>8</td>
<td>31 (30, 32)</td>
<td>34 (34, 35)</td>
<td>36 (35, 37)</td>
</tr>
<tr>
<td>9</td>
<td>33 (31, 34)</td>
<td>na</td>
<td>37 (36, 39)</td>
</tr>
<tr>
<td>10</td>
<td>35 (34, 36)</td>
<td>&gt;37</td>
<td>&gt;40 (39, 41)</td>
</tr>
<tr>
<td>11</td>
<td>33 (32, 35)</td>
<td>na</td>
<td>38 (36, 40)</td>
</tr>
<tr>
<td>12</td>
<td>36 (35, 38)</td>
<td>&gt;37</td>
<td>&gt;40</td>
</tr>
<tr>
<td>13</td>
<td>34 (32, 36)</td>
<td>na</td>
<td>39 (37, 40)</td>
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<tr>
<td>14</td>
<td>&gt;37 (36, 38)</td>
<td>&gt;37</td>
<td>&gt;40</td>
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<tr>
<td>15</td>
<td>&gt;37 (36, 40)</td>
<td>na</td>
<td>&gt;40</td>
</tr>
<tr>
<td>16</td>
<td>&gt;37</td>
<td>&gt;37</td>
<td>&gt;40</td>
</tr>
</tbody>
</table>

\(^a\) Real-time reverse-transcription polymerase chain reaction (rRT-PCR), virus isolation and "Point of care" (POC) rapid antigen test (VetScan™, Abaxis Inc.)

\(^b\) Oral fluid (OF) and nasal swab (NS) samples

\(^c\) No sample collected
Table 6. Influenza A virus rRT-PCR hemagglutinin subtyping (H1 or H3) results for rRT-PCR IAV-positive samples

<table>
<thead>
<tr>
<th>Laboratory A</th>
<th>Laboratory B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oral fluids&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Nasal swabs&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>DPI</td>
<td>N</td>
</tr>
<tr>
<td>1</td>
<td>18</td>
</tr>
<tr>
<td>2</td>
<td>20</td>
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<td>3</td>
<td>18</td>
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<td>15</td>
<td>4</td>
</tr>
<tr>
<td>16</td>
<td>3</td>
</tr>
</tbody>
</table>

<sup>a</sup> “N” gives the number of rRT-PCR matrix gene-positive samples by DPI. Match (M) indicates the number of samples correctly identified by subtype, e.g. H1 or H3. Mis-match (MM) indicates the number of samples in which the subtype was incorrectly identified and undetermined (UD) indicates the number of samples in which it was not possible to achieve subtype identity.

<sup>b</sup> No nasal swab samples collected on DPI 7, 9, 11, 13, or 15.
Figure 1. Probability of detecting IAV in pen-based OF (a) or individual NS (b) from unvaccinated pigs by assay over time (*"Point of care" (POC) rapid antigen test (VetScan™, Abaxis Inc.)
Figure 2. Probability of IAV detection in pen-based OF (a) or individual NS (b) from vaccinated pigs by assay over time ("Point of care" (POC) rapid antigen test (VetScan™, Abaxis Inc.))
Figure 3. Probability of detecting IAV in OF (a) and NS (b) by rRT-PCR by laboratory in unvaccinated and vaccinated pigs over time
CHAPTER 4. EVALUATION OF SCREENING ASSAYS FOR THE DETECTION OF INFLUENZA A VIRUS SERUM ANTIBODIES IN SWINE

Christa K. Goodell, John Prickett, Apisit Kittawornrat, John Johnson, Jianqiang Zhang, Chong Wang, Jeffrey J. Zimmerman

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ABSTRACT

Increased surveillance of influenza A virus (IAV) infections in human and swine populations is mandated by public health and animal health concerns. Antibody assays have proven useful in previous surveillance programs because antibodies provide a record of prior exposure and the technology is inexpensive. The objective of this research was to compare the performance of influenza serum antibody assays using samples collected from pigs (vaccinated or unvaccinated) inoculated with either A/Swine/OH/511445/2007 γ H1N1 virus or A/Swine/Illinois/02907/2009 Cluster IV H3N2 virus and followed for 42 days. Weekly serum samples were tested for anti-IAV antibodies using homologous and heterologous hemagglutination inhibition (HI) assays, commercial swine influenza H1N1 and H3N2 indirect ELISAs, and a commercial influenza nucleoprotein (NP) blocking ELISA assay. The homologous HIs showed 100% diagnostic sensitivity, but largely failed to detect infection with the heterologous virus. With diagnostic sensitivities of 1.4% and 4.9%, respectively, the H1N1 and H3N2 indirect ELISAs were ineffective at detecting IAV antibodies in swine infected with the contemporary influenza viruses used in the study. At a cut-off of S:N ≤ 0.60, the sensitivity and specificity of the NP blocking ELISA assay were estimated at 95.5% and 99.6%, respectively. Statistically significant factors which affected S:N responses include vaccination status, inoculum (virus subtype), day post inoculation, and the interactions between those factors (p < 0.0001). Serum antibodies against NP provide an ideal universal diagnostic screening target and could provide a cost-effective approach for the detection and surveillance of IAV infections in swine populations.
INTRODUCTION

Influenza A virus (IAV) is a concern both as a public health issue and a significant pathogen of swine (Alexander, 1982; Kothalawala et al., 2006; Lorusso et al., 2012; Myers et al., 2007; Subbarao et al., 2006; Vincent et al., 2008; Webster et al., 1992; Zhu et al., 2011). The most recent stimulus to improve our understanding of IAV in human and swine populations through surveillance was the 2009 influenza pandemic (Garten et al., 2009). Like other pandemics, the H1N1pdm09 (pH1N1) transmitted well and quickly between humans. The first cases (March 2009) were in persons living in Mexico (CDC, 2009; Perez-Padilla et al., 2009) followed rapidly by reports in humans in the U.S. and throughout the world (WHO map 2009). Unique from other pandemics, interspecies transmission occurred quickly. The first swine case of pH1N1 was reported in May 2009, apparently introduced into the herd by a caretaker infected with the virus (Howden et al., 2009). Subsequently, many transmission events among and between the human and swine populations were documented (Lange et al., 2009; Nelson et al., 2012; Weingartl et al., 2010).

Pappaioanou and Gramer (2010) stated, "With the continual circulation and interspecies transmission of human, swine and avian influenza viruses in countries around the world, there are calls for strengthening influenza surveillance in pigs, birds and other animals to aid in monitoring and assessing the risk of future pandemic virus emergence involving different species." The majority of programs currently collecting data on IAV in populations rely on testing of convenience samples from birds (wild and domestic) or humans using antibody, virus isolation, and/or RT-PCR assays (Broor et al., 2012; Escorcia et al., 2012; Hoye et al., 2010; Pawar et al., 2012; Stöhr, 2003; Thinh et al., 2012; Petruccelli et al., 2010; WHO, 2011). Recently, routine reporting of influenza in swine was initiated in the U.S. through veterinary diagnostic laboratories in cooperation with herd veterinarians (USDA, 2010).

In veterinary medicine, IAV surveillance on farms is justified by its role as an economically important pathogen of swine and a significant component of the "porcine respiratory disease complex". A survey of commercial swine production companies producing almost half the market pigs in the US ranked IAV as among the most economically important pathogens of
nursery, finishing, and breeding animals (Holtcamp and Rotto, 2007 a,b).

In public health, IAV surveillance in swine populations is justified by the fact that IAV is communicable among humans and swine (zoonotic). Examples of the transmission of IAV from pigs to human are common. At an Ohio fair people became ill with influenza symptoms after an influenza virus infected 100% of the show pigs (Killian et al., 2012) and triple-reassortant swine H1N1 influenza viruses were subsequently identified in both pigs and people. In a contemporary study following swine workers prospectively over a period of two years, Terebuh et al., (2009) concluded that workers had “serologic evidence for infection with both swine and human influenza viruses and were exposed to diverse influenza virus strains circulating in pigs.” Transmission of IAV from humans to pigs is likely as common, but often unrecognized. Nelson et al. (2012) identified at least 23 separate human-to-pig transmission events (H1 or H3) since 1990 and at least 49 human-to-pig pH1N1 transmission events during 2009-2011. Shu et al. (1996) found serologic evidence that farm pigs in China were infected by the same human influenza viruses circulating in caretakers' families. Rith et al. (2012) investigated the seroprevalence of human IAVs in swine populations from 2006-2010 and found that 15% of the pigs were positive for antibodies to the human isolates circulating at the time.

Although detection of nucleic acid via PCR is useful in identifying acute infections, antibody-based assays have proven to be an efficient and cost-effective tool for the surveillance of high-impact diseases, e.g., Aujeszky's disease in swine (Foti et al., 2008; Seigel and Weigel, 1999; Lehman et al., 1993; Lehman et al., 1994; Wyckoff et al., 2009) and human immunodeficiency virus (HIV) in humans (Bassett et al 2010; Clark et al., 2010; Clauss et al., 2011; Delaney et al., 2011; Edeh and Spalding, 2000; Lee et al., 2011; Mortimer and Parry, 1994). Antibody surveillance, less sensitive to sample collection timing, is more useful for detecting IAV exposure than PCR-based assays. In mammals, screening for influenza antibodies is currently not common practice, because an assay capable of screening for infection by diverse influenza subtypes has not been implemented. Therefore, the purpose of this study was to compare currently available IAV serum antibody
assays using samples collected over time from swine of known vaccination and infection status.

MATERIALS AND METHODS

Experimental design
The detection of influenza A virus (IAV) serum antibodies over time was evaluated in samples from IAV vaccinated and unvaccinated pigs inoculated with subtypes H1N1 or H3N2 and followed for 42 days post inoculation (DPI). Serum samples were collected at weekly intervals and tested on four serum antibody assays: hemagglutination inhibition, two commercial indirect ELISAs\textsuperscript{a,b} and a commercial IAV nucleoprotein (NP) blocking ELISA\textsuperscript{c}. The influenza antibody response data were analyzed for the effect of virus subtype, vaccination, time, and their interactions. Receiver operator curve (ROC) analyses were performed to estimate and compare assay performance.

Influenza A viruses
Isolate A/Swine/OH/511445/2007 γ H1N1 virus (provided by Dr. Amy Vincent, USDA National Animal Disease Center, Ames, IA) was recovered from an influenza outbreak at a county fair in Ohio. A/Swine/Illinois/02907/2009 Cluster IV H3N2 virus (provided by Dr. Marie Gramer, University of Minnesota, St. Paul, MN) was isolated from clinically-affected finishing pigs in Illinois. Both viruses were propagated in Dr. Vincent's laboratory on Madin-Darby Canine Kidney cells to achieve virus concentration of approximately $1 \times 10^{6.5}$ median tissue culture infectious dose (TCID\textsubscript{50}) per ml.

Animals and animal care
The study was conducted under the approval of, and in compliance with, the guidelines of the Iowa State University (ISU) Institutional Animal Care Use Committee (#11-09-6834-S) and the Institutional Biosafety Committee (09-I-0028-A). Iowa State University is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).
Health status  IAV-negative piglets (n = 82) were obtained from one 600 sow farm at ~21 days of age and an average weight of 6.26 kg (13.8 pounds). The source herd was considered negative for IAV, porcine reproductive and respiratory syndrome virus (PRRSV), and *Mycoplasma hyopneumoniae* on the basis of historic serologic herd monitoring. Additional serologic testing of sows in breeding and gestation phases and non-study piglets on sows was conducted 10 days prior to receipt of the study piglets, with sow sample numbers based on 90% confidence of detecting a 10% prevalence. Piglets’ serum samples collected at this time were also assayed for PRRSV infection by reverse-transcriptase polymerase chain reaction (RT-PCR) testing of pooled serum samples (5 pigs per pool).

Diet  Pigs were fed standard, commercially-formulated diets throughout the study. After weaning, piglets were fed a commercially-formulated, pelleted, whey-based, starter diet medicated with chlortetracycline (35g/ton) and tiamulin (400g/ton) (United Farmers Coop, Lesueur, MN). At approximately 49 days of age, and through the duration of the study, they were fed commercial, non-medicated corn:soy meal diets (United Farmers Coop, Lesueur, MN; Heartland Coop, Des Moines, IA).

PCV2 vaccination  The source farm routinely vaccinated piglets against porcine circovirus type 2 (PCV2) at 5 and 21 days of age using a commercial PCV2 vaccine. On DPI -28, i.e., at ~36 days of age, all piglets were revaccinated against PCV2 according to label instructions.

Study timeline and treatments  On DPI -43, the 82 ~21-day-old piglets were moved from the sow farm into one room of a disinfected isolation facility. On DPI -42, each animal was visually inspected and ear tagged. Serum samples were collected on DPI -42 (and subsequently on DPIs -21, -7, and 0) and tested for antibodies against IAV, PRRSV, and *M. hyopneumoniae* to assure continued negative status of the group.

Pigs were randomized to one of six 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. Since the
pigs were housed as one group in the isolation facility, ear tag color was used to differentiate pigs in IAV vaccinated (n = 28) and nonvaccinated (n = 54) treatment groups. Vaccinated pigs were intramuscularly administered a trivalent commercial IAV vaccine\textsuperscript{g} [A/Swine/Iowa/110600/00 $\gamma$ (H1N1), A/Swine/North Carolina/031/05 $\delta$ (H1N1), and A/Swine/Missouri/069/05(H3N2)] according to label instructions on DPI -42 and DPI -21. The $\gamma$ H1N1 and Cluster 4 H3N2 components of the vaccine were 95.4\% and 98.4\% homologous to the HA amino acid sequences of the H1N1 and H3N2 viruses used to inoculate pigs and in the hemagglutination inhibition assays described below.

On DPI -10, the animals were moved to the ISU Livestock Infectious Disease Isolation Facility and allowed to acclimate for ten days. Animals were housed by IAV inoculant group (negative control, H1N1, or H3N2), with 3 or 4 pigs per pen. Vaccinates were housed with their inoculant group, but were penned separately from non-vaccinates. The control group was housed in one room in four pens configured in a 2 x 2 arrangement and constructed with gates that permitted nose-to-nose contact. The H1N1 and H3N2 inoculant groups were each housed in 2 rooms; one room of 5 pens and one room of 6 pens for each group. These pens were constructed of solid aluminum panels that precluded pig contact between pens.

Pigs were observed daily throughout the experiment. Manual contact was used to stimulate movement or assess lethargy, if animals were reluctant to rise. Individual animal weights were obtained at DPI -42 and DPI 0 using portable electronic scales\textsuperscript{h} as a general assessment of health and pig uniformity.

On DPI 0, animals in inoculant groups H1N1 and H3N2 were intratracheally administered 2 ml of a solution containing $1 \times 10^{6.5}$ TCID\textsubscript{50} per ml of either A/Swine/OH/511445/2007 $\gamma$ H1N1 or A/Swine/Illinois/02907/2009 cluster IV H3N2. A portion of each inoculum was stored (-80°C) for back-titration.
Serum samples
Serum samples were collected on DPI -42, -21, -7, 0, 7, 14, 21, 28, 35, and 42. Blood samples were drawn from the jugular vein or cranial vena cava using a single-use blood collection system and serum separation tubes. Samples were centrifuged at 1800 x g at 4°C, after which sera were aliquotted into 5 ml tubes and stored at -20°C until tested.

Diagnostic testing
To verify the status of the group prior to inoculation with IAV, serum samples collected on DPI -42, -21, -7, 0, 7 were tested after collection for evidence of infection with PRRSV (antibody ELISA), and-real-time reverse-transcriptase polymerase chain reaction (rRT-PCR), M. hyopneumoniae (antibody ELISA), and IAV (antibody ELISA). At the conclusion of the experiment, serum samples collected on DPI -42, -21, -7, 0, 7, 14, 21, 28, 35, and 42 were completely randomized and tested with four IAV serum antibody assays: hemagglutination inhibition, two indirect ELISAs, and an IAV NP blocking ELISA.

Laboratory quality assurance Serologic assays were performed at the ISU Veterinary Diagnostic Laboratory, an American Association of Veterinary Laboratory Diagnostics (AAVLD) accredited laboratory. Quality assurance procedures included routine calibration, monitoring, and maintenance of all laboratory instruments and equipment. All technical personnel participated in pipette training, in-house check-test panels, and written examinations on the performance of serologic testing.

Antibody detection Indirect antibody ELISAs licensed for testing swine serum were performed according to the manufacturer’s instruction and analyzed using the manufacturer's software. Negative and positive controls provided by the manufacturer were run on all plates and the performance of each plate was validated using the manufacturer's software. As an additional quality control step, in-house negative and positive controls were run on each M. hyopneumoniae ELISA plate and four in-house controls (negative, low, intermediate, and high positives) were run on each PRRSV ELISA plate. In-house and kit control results were analyzed using statistical process control software. Indirect ELISA results were expressed
as S/P (sample/kit positive control) values and samples with S/P values ≥ 0.40 were considered positive.

Serum samples were also tested for IAV antibody using an NP blocking ELISA<sup>c</sup>. The assay is licensed for use in avian species (chicken, turkey, duck, ostrich, and goose), but was shown to possess excellent diagnostic performance in swine and is performed routinely in the ISU-VDL (Ciacci-Zanella et al., 2010). The assay was conducted according to the manufacturer's instructions and the laboratory's standard operating procedure (Boesenberg, 2012). Consistent with the other ELISAs, quality control included statistical process control charting of in-house negative and positive controls. Data management and calculations were performed using the software provided by the manufacturer. Results were reported as S/N (sample/kit negative control) ratios. A receiver operator characteristic curve was used to determine the optimized cut-off for the NP blocking ELISA in swine serum.

Homologous hemagglutination inhibition (HI) assays were performed using influenza virus isolates A/Swine/OH/511445/2007 γ H1N1 and A/Swine/Illinois/02907/2009 Cluster IV H3N2 according to standardized procedures at the Iowa State University Diagnostic Laboratory (Block, 2011a,b; Block, 2012).

**PRRSV real-time reverse-transcriptase polymerase chain reaction (rRT-PCR)**  Serum samples were pooled in groups of 4 or 5 for testing. Nucleic acid was extracted<sup>i</sup> and purified<sup>i</sup> according to manufacturer’s recommendations. PRRSV rRT-PCR was performed using NA and EU PRRSV Reagents<sup>i</sup> with master mix made following manufacturer’s recommendations. An internal RNA control<sup>i</sup> was included at the master mix step. The reactions were run<sup>i</sup> with cycling conditions as follows: 45°C for 10 minutes, then 95°C for 10 minutes, followed by 40 cycles of 97°C for 2 sec, and 60°C for 40 sec. The instrument was run in “fast” mode and fluorescence data was collected during the 60°C annealing/extension stage. Analysis was performed using the auto baseline setting, with thresholds for NA and EU PRRSV set at 0.2 and 0.05, respectively. A sample was considered positive for the respective PRRSV subtypes if it yielded a cycle threshold (Ct) of ≤ 37.
Data analysis
For HI results, antibody titers $\geq 1:20$ were transformed by dividing the reciprocal by 10 and taking the $\log_2$. The geometric mean antibody titers (GMT) of a group were calculated as the antilog$_2$ of the arithmetic mean of each group x 10 (Thrusfield, 2005). Least square (LS) mean values and 95% confidence intervals (95% CI) for ELISA S/P or S/N values were calculated by treatment group by day post inoculation (DPI) for indirect and NP blocking ELISA’s, respectively.

The diagnostic performance of ELISA and HI assays were evaluated using receiver operator characteristics (ROC) analyses$^\text{v}$. ROC analyses were based on test results from expected negative samples (n = 279) collected from unvaccinated and uninoculated pigs (DPI -42 through DPI 42) and results from expected positive samples (n = 288) collected from UV$_{H1}$ and UV$_{H3}$ at DPI $\geq$ 7. Two ROC curves were calculated for subtype-specific assays: a "cumulative" ROC based on all expected positive samples (n = 288) and a "homologous" ROC using the subset of expected positive samples (n = 144) from pigs inoculated with the virus homologous to the assay. Area under the curve (AUC) was calculated using the trapezoidal rule (Bamber, 1975).

A repeated measures analysis of variance (R-ANOVA) model$^\text{u}$ using a Type III test for fixed effects was used to analyze the association between the H1N1 and H3N2 HI results. Likewise, this approach was used to analyze the outcome of the NP blocking ELISA assay in relation to the fixed effects of vaccination status, inoculum (virus subtype), inoculation status (inoculated/not inoculated), days post inoculation (DPI), and the interactions of these factors, where individual pig was the unit of repeated measures. Tukey-Kramer t-tests were used for pair-wise comparisons of ELISA responses between treatment groups by day.

RESULTS

General observations All negative and positive kit controls (ELISA$^{a,b,k,l}$) and in-house (ELISA$^{c,k,l}$) control values were within normal limits and met statistical process control performance criteria. All animals were negative (serum antibody and RT-PCR) for M.
hyopneumoniae and PRRSV on DPI -42, -21, -7, 0, and 7. At DPI 0, all animals appeared clinically normal and no significant difference in pig weight by treatment group was observed.

Following inoculation, titration of A/Swine/OH/511445/2007 γ H1N1 and A/Swine/Illinois/02907/2009 Cluster IV H3N2 yielded concentration estimates of $1 \times 10^{6.5}$ and $1 \times 10^{5.7}$ TCID₅₀/ml, respectively. Pigs were reluctant to move on DPI 1, but not on DPI 2 or later. One pig was treated for polyarthritis with ceftiofur sodium (4mg/kg) on DPI 4, but no animals required treatment for respiratory disease and no animals were removed from the study.

**Hemagglutination Inhibition**  An H3N2 HI response of 1:20 was detected in one of 82 pigs tested on DPI -42. On DPI -21, -7, and 0, all unvaccinated animals tested < 1:20 in the H3N2 HI assay. Two unvaccinated pigs had H1N1 HI responses of 1:20 on DPI -21 and one unvaccinated pig on DPI -7. Among vaccinates, 18 of 28 animals (64%) were H3N2 HI antibody positive (≥ 1:20) on DPI -21 (GMT 1:25), and 27 of 28 (GMT 1:207) on DPI -7. Similarly, 14 of 28 vaccinates (50%) were H1N1 HI positive on DPI -21 (GMT 1:26) and all 28 were positive (GMT 1:249) on DPI -7.

Quantitative HI results for DPI 0 to 42 are reported in Table 2. UV_CTRL were negative (< 1:20) through DPI 42. When unvaccinated pigs (UV_H1 and UV_H3) were tested on the homologous HI assay, IAV antibody was detected in all pigs by DPI 7, with the highest antibody titers detected on DPI 14. When vaccinated pigs (V_H1, V_H3) were tested on the homologous HI, peak antibody titers were observed in the H1N1 assay on DPI 14 vs. DPI 0 on the H3N2 assay. When tested on the heterologous HI assay, UV_H1 were negative on the H3N2 HI, whereas low H1N1 HI antibody titers were detected in a few UV_H3, i.e., 5 pigs at DPI 7 (GMT 1:23) and one pig at DPI 14 (GMT 1:20). Qualitative HI results are reported in Table 3. Throughout the study, all 28 vaccinates (V_CTRL, V_H1, V_H3) were H1N1 HI positive and 27 of 28 were H3N2 HI positive (Table 3). However, GMT in V_CTRL and V_H1 and V_H3 declined quickly on the heterologous HI.
The homologous ROC analyses of the H1N1 and H3N2 HI assays estimated the area under the curve (AUC) at 1.0 for each. Using a cutoff of $\geq 1:20$, the resulting sensitivities and specificities were estimated at 100% (Table 4). In contrast, the cumulative ROCs estimated the AUCs at 0.76 and 0.75 for the H1N1 and H3N2 HI assays, respectively, with sensitivities and specificities of approximately 50% and 100% for each.

**Indirect ELISA antibody detection** All animals tested negative ($S/P < 0.40$) on the two IAV indirect antibody ELISA assays$^{a,b}$ on DPI -42, and -21. On DPI -7, 11 of 28 (39%) vaccinates ($V_{CTRL}$, $V_{H1}$ and $V_{H3}$) were positive on the H3N2 indirect ELISA$^{b}$ (LS mean $S/P = 0.31$ (95% CI: 0.27, 0.35)). In contrast, 2 of 28 vaccinates (7%) were positive on the H1N1 indirect ELISA$^a$ (LS mean $S/P = 0.14$ (95% CI: 0.10, 0.17)). On DPI 0, the mean ELISA$^{a,b}$ $S/P$ values were 0.0 for $UV_{CTRL}$, $UV_{H1}$ and $UV_{H3}$ and ranged between 0.13 and 0.35 in $V_{CTRL}$, $V_{H1}$ and $V_{H3}$ (Table 2). Thereafter, the mean ELISA$^{a,b}$ $S/P$ values for $UV_{H1}$ and $UV_{H3}$ ranged from 0.0 to 0.22 for both homologous and heterologous groups. IAV-inoculated pigs were rarely positive unless they were also vaccinated (Table 3).

On the H1N1 ELISA$^a$, 73 to 100% of $V_{H1}$ tested positive between DPI 7 to 42 (Tables 2 and 3). In contrast, 0 to 27% of $V_{H3}$ were positive on the same assay. On the H3N2 ELISA$^b$, 45 to 82% of $V_{H3}$ were positive between DPI 7 to 42; whereas, 9 to 55% of $V_{H1}$ were positive. The homologous ROC analyses for the H1N1 and H3N2 indirect ELISA$^{a,b}$ assays estimated the AUC for both assays at 0.936 (Table 4). Using the manufacturer's recommended cutoff ($S/P \geq 0.40$), the sensitivities of the assays were 1.4% and 4.9% for the H1N1 and H3N2 ELISA, respectively, with specificities of approximately 100% for each. The cumulative ROC estimated the AUCs at 0.844 (H1N1) and 0.872 (H3N2) with sensitivities of 0.7% and 2.8% respectively.

**NP Blocking ELISA antibody detection** Results (LS means) are reported as $S/N$ (sample/kit negative control) ratios by treatment group and DPI in Table 2. On DPI -42, the mean $S/N$ response was 0.89 (95% CI: 0.86, 0.93). On DPI 0, the mean $S/N$ response of unvaccinated pigs ($UV_{CTRL}$, $UV_{H1}$ and $UV_{H3}$) was 0.85 (95% CI: 0.82, 0.89) vs. 0.69 (95% CI: (0.64, 0.75)
in vaccinated pigs (V\textsubscript{CTRL}, V\textsubscript{H1} and V\textsubscript{H3}).

ROC analyses based on H1N1, H3N2, and cumulative data estimated AUCs of ~0.99. Using an optimized cutoff of S/N ≤ 0.60, this resulted in sensitivities of 96.5%, 94.4%, and 95.5%, respectively, and specificities of 99.6% (Table 4, Figure 1). At a cutoff of S/N ≤ 0.60, control pigs remained negative throughout the study, although the mean S/N of V\textsubscript{CTRL} and UV\textsubscript{CTRL} were significantly different on DPI 0 and 28. The LS mean S/N values of UV\textsubscript{H1}, V\textsubscript{H1}, UV\textsubscript{H3} and V\textsubscript{H3} were positive by DPI 7. At each sampling after DPI 0, 64 to 68 of the 70 inoculated pigs were positive on the NP blocking ELISA. Throughout the study and regardless of virus subtype, the NP blocking ELISA detected influenza antibodies in 88 to 100% (21 to 24) of the animals in UV\textsubscript{H1} and UV\textsubscript{H3} groups and 82 to 100% (9 to 11) of the pig in V\textsubscript{H1} and V\textsubscript{H3} groups (Table 3). A repeated measures ANOVA determined that vaccination status, inoculum (virus subtype), DPI, and the interactions of DPI*vaccination status and DPI*inoculum*vaccination status were significant to the NP blocking ELISA S/N responses (p < 0.0001). As shown in Table 5, when the test of effects (for DPI*inoculum*vaccination status) was sliced by DPI, all sampling points except DPI -21 were significant when compared to the mean value on DPI -42 (p < 0.0001).

DISCUSSION

By definition, monitoring is “An ongoing effort at assessing the health and disease status of a given population” (Salman, 2003). Monitoring can be for a specific agent, disease, or other health-related target. The breadth of the program (size or area) is determined by its objective(s). Most commonly in animal populations, the purpose of monitoring is to detect disease agents and track changes in prevalence, incidence, and/or geographic distribution. Surveillance is monitoring with a planned response to a pre-assigned trigger, i.e., an action is taken to mitigate the impact of disease or the pathogen (Salman, 2003). Surveillance is considered, “… a more intensive form of data recording than monitoring, so action can be taken to control disease” (Thrusfield, 2005). Salman (2003) defines the three components of a surveillance system as: (1) a monitoring system, (2) a threshold above which action will be taken, and (3) a pre-determined intervention strategy. Effective monitoring systems must
address sampling framework, sample specimen type, sample size, sampling frequency, and detection methods. Within the context of IAV monitoring, the objective of this study was to compare contemporary IAV serum antibody assays (hemagglutination inhibition, indirect ELISA, and a blocking ELISA) using samples from pigs of known IAV status. Because influenza vaccines are commonly used in swine populations, a vaccinated group was included to quantify the effect of vaccination on antibody response and test performance.

The hemagglutination inhibition (HI) assay is based on the ability of IAV to agglutinate red blood cells (RBCs) via attachment of the viral hemagglutinin to sialic acids, e.g., N-acetylneuraminic acid, on RBCs (Paulson et al., 1979; Rogers et al., 1986). Hirst (1941, 1942) was the first to report that IAV agglutinated chicken RBCs and that the reaction was inhibited in the presence of anti-IAV serum antibodies; thereby describing the mechanism of the hemagglutination-inhibition (HI) serum antibody assay. Conceptually simple, subsequent work showed that the strength of the HI reaction was affected both by differences among animal species in the density of viral receptors on RBCs (Kumari et al., 2007; Ito et al. 1997) and differences in the affinity of IAV isolates for the specific receptors expressed on the RBCs (Cheng et al., 2012; Ito et al., 1997; Makkoch et al., 2012; Rogers and Paulson, 1983; Rogers et al, 1986; Rogers and D’Souza, 1989; Stephenson et al., 2003; Wibawa et al., 2012). Further complicating the issue, viral characteristics are not necessarily stable, e.g., laboratory passage of IAV isolates can change viral receptor specificity (Bogdan et al., 1992; Daniels et al., 1984; Lin et al., 2010; Rogers and Paulson, 1983). Cumulatively, these sources of variability have been shown to markedly affect HI reproducibility and repeatability (Rossow et al., 2003; Stephenson et al., 2009; Wagner et al., 2012; Wood et al., 2012; Wood et al., 2011a; Wood et al., 2011b).

The HI results in this study were consistent with previous reports that heterology between the virus used in the HI assay and the virus that induced antibodies in the pig resulted in a loss of diagnostic sensitivity (Table 4) (Gramer et al., 2007; Long et al., 2004; Leuwerke et al., 2008; Yoon et al., 2004). Among the animals in groups UV_{H1} (n = 24) and UV_{H3} (n = 24) all animals were positive by DPI 7 on the homologous HI assay, but only 5 were positive on the
heterologous HI assay (Table 3). This relationship was obscured by vaccination. That is, all 28 vaccinated animals (VCTRL, VHI, and VH3) were HI positive on DPI 0 and 27 of 28 were positive throughout the study. This response may reflect the fact that the trivalent vaccine included A/Swine/Iowa/110600/00 γ (H1N1) and A/Swine/Missouri/069/05 cluster IV (H3N2), which respectively shared 95.4% and 98.4% HA amino acid homology to the viruses used in the two HI assays. In addition, differences were also observed in the magnitude of the HI antibody titer response between pigs inoculated with H1N1 versus H3N2 viruses (Table 2). This difference may be attributed to variability inherent in the HI assay, as discussed in the previous paragraph. Cumulatively these results reinforced the significant weaknesses of the HI for both routine diagnostics and surveillance.

The H1N1 and H3N2 indirect serum antibody ELISAs evaluated in this study were licensed by the USDA Center for Veterinary Biologics in 2001 (USDA, 2001) and 2005 (USDA, 2005), respectively. These tests detect anti-H1N1 or anti-H3N2 serum antibody by visualizing the presence of antibody-antigen complexes using a colorimetric reaction and results are calculated as sample:positive (S/P) ratios. The advantage offered by the ELISA format over HI assays is rapid throughput, higher repeatability, and better quality control in the diagnostic laboratory. However, both assays were ineffective at detecting IAV antibodies in swine infected with the contemporary influenza viruses used in the study. These results are consistent with other recent reports (Barbe et al., 2009; Leuwerke et al., 2008; Yoon 2006c).

NP is the second most abundant protein in the IAV virion and is propagated in large quantities in infected cells (Webster et al., 1992). NP is considered to be highly genetically conserved and amino acid identity in mammals is generally > 90% (Gorman et al., 1990). NP stimulates both cell mediated and humoral immunity (Carragher et al., 2008; Sukeno et al., 1979).

The blocking ELISA detects serum antibodies against the internal IAV NP, an integral intranuclear viral protein involved in IAV genome replication and transcription. Antibodies
to NP present in serum bind to the avian NP antigen-coated substrate of the assay wells and block labeled monoclonal antibody from binding. The blocking format does not require species-specific reagents and, for this reason, the NP ELISA is currently used to detect IAV serum antibodies in a variety of species, including swine (Brown et al., 2009; Ciacci-Zanella et al., 2010; Tse et al., 2012; Kittleberger et al., 2011). At a cut-off of S:N ≤ 0.60, the sensitivity and specificity of the assay were equivalent to the homologous HIs and better than the heterologous HIs. Cumulatively, the sensitivity and specificity of the NP blocking ELISA assay were estimated at 95.5% and 99.6%, respectively (Table 4); a level of performance consistent with previous reports (Brown et al., 2009; Brown et al., 2010a; Brown et al., 2010b; Ciacci-Zanella et al., 2012; Kittelberger et al 2011; Lebarbenchon et al., 2012; Tse et al., 2012). In contrast to HI assays, the ELISA format provides the advantage of improved repeatability (within laboratory) and reproducibility (between laboratory).

Seven of 28 vaccinated animals were positive on the NP blocking ELISA on DPI 0 (Table 3). In addition, vaccination affected the magnitude and/or duration of the S/N response over the course of the study. That is, the mean S/N was significantly lower in vaccinates vs. non-vaccinates at DPI 0; was equivalent from DPI 21 to 35, and was significantly higher in vaccinates vs. non-vaccinates at DPI 42 (Table 5). While vaccination affected the S/N response, the data did not support the use of the NP ELISA for monitoring IAV vaccination compliance or for differentiation of vaccinated and IAV-infected animals.

This study confirmed the lack of utility of the HI assay for influenza antibody detection because of the biological vagaries of the assay. Similarly, the current H1N1 and H3N2 indirect ELISAs evaluated in the study were shown to be unable to detect antibodies against contemporary IAVs circulating in swine. In contrast, NP is produced in abundance, is immunogenic, and is highly conserved among IAVs. Antibodies against NP provide an ideal universal diagnostic screening target and, as this study showed, could provide a cost effective approach for the detection and surveillance of IAV infections in swine populations.
DECLARATION OF CONFLICTING INTERESTS

The authors declare no conflicting interests with respect to their authorship or the publication of this article.

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 SOURCES AND MANUFACTURERS

a. IDEXX SIV H1N1 Ab Test, IDEXX Laboratories, Inc., Westbrook ME.
b. IDEXX SIV H3N2 Ab Test, IDEXX Laboratories, Inc., Westbrook, ME.
c. IDEXX AI MultiS-Screen Ab Test, IDEXX Laboratories, Inc., Westbrook, ME.
d. Denagard®, Novartis Animal Health US, Inc., Greensboro, NC.
e. Circumvent®PCV Intervet/Shering-Plough Animal Health, Millsboro, DE USA.
f. Suavaxyn® PCV2 One-Dose, Fort Dodge Animal Health, New York NY USA.
g. Flu-Sure® XP, Pfizer Animal Health, Madison, NJ.
h. Becton Dickson, Franklin Lakes, NJ, USA.
i. Kendall, Mansfield, MA, USA.
j. BD Falcon™, Franklin Lakes, NJ, USA.
k. IDEXX PRRS X3 Ab Test., IDEXX Laboratories, Inc., Westbrook, ME.
l. IDEXX M hyo Ab Test, IDEXX Laboratories, Inc., Westbrook, ME.
m. Artel, Westbrook, ME.
n. xChek®, IDEXX Laboratories, Inc.
o. Northwest Analytic SPC, Portland Oregon.
p. MagMax™ 96 Viral RNA Isolation Kit, Applied Biosystems®, Foster City, CA.
q. Kingfisher® 96, Thermo Electron, West Palm Beach, FL.
r. TaqMan® NA and EU PRRSV Reagents, Applied Biosystems®, Foster City, CA.
s. Xeno®, Applied Biosystems®, Foster City, CA.
t. 7500 Fast Instrument, Applied Biosystems®, Foster City, CA.
v. MedCalc® v 12.2.1.0, MedCalc software, Mariakerke, Belgium.
w. Fisher Scientific traceable noncontact IR thermometer, Pittsburg, PA, USA.
x. Siltec® WS500 Electronic Weighing Scale Bradford, MA, USA.
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CDC: April 30, 2009, Outbreak of Swine-Origin Influenza A (H1N1) Virus Infection --- Mexico, March--April 2009. MMR 58(Dispatch):1-3


USDA: January 10, 2001, Center for Veterinary Biologics Notice No. 01-01.

USDA: April 18, 2005, Center for Veterinary Biologics Notice No. 05-09.


WHO. May 20, 2009. New Influenza A(H1N1), Number of Laboratory confirmed cases and deaths as reported to WHO. http://www.who.int/csr/don/h1n1_20090520_0600.jpg


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

\(^*\text{Vaccinated on DPI -42 and -21 with a trivalent, inactivated influenza vaccine}\)
Table 2. Summary of antibody assay results: LS mean (ELISAs) or GMT (HIs) of animals by vaccination status\(^a\) and IAV inoculant over time.

<table>
<thead>
<tr>
<th>Assay by treatment group</th>
<th>No. of pigs</th>
<th>DPI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>UV(_{CTRL})</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>V(_{CTRL})</td>
<td>6</td>
<td>201</td>
</tr>
<tr>
<td>UV(_{H1})</td>
<td>24</td>
<td>0</td>
</tr>
<tr>
<td>V(_{H1})</td>
<td>11</td>
<td>160</td>
</tr>
<tr>
<td>UV(_{H3})</td>
<td>24</td>
<td>0</td>
</tr>
<tr>
<td>V(_{H3})</td>
<td>11</td>
<td>193</td>
</tr>
<tr>
<td>UV(_{CTRL})</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>V(_{CTRL})</td>
<td>6</td>
<td>142</td>
</tr>
<tr>
<td>UV(_{H1})</td>
<td>24</td>
<td>0</td>
</tr>
<tr>
<td>V(_{H1})</td>
<td>11</td>
<td>109</td>
</tr>
<tr>
<td>UV(_{H3})</td>
<td>24</td>
<td>0</td>
</tr>
<tr>
<td>V(_{H3})</td>
<td>11</td>
<td>205</td>
</tr>
<tr>
<td>UV(_{CTRL})</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>V(_{CTRL})</td>
<td>6</td>
<td>0.13(^b)</td>
</tr>
<tr>
<td>UV(_{H1})</td>
<td>24</td>
<td>0</td>
</tr>
<tr>
<td>V(_{H1})</td>
<td>11</td>
<td>0.15(^b)</td>
</tr>
<tr>
<td>UV(_{H3})</td>
<td>24</td>
<td>0</td>
</tr>
<tr>
<td>V(_{H3})</td>
<td>11</td>
<td>0.18(^b)</td>
</tr>
<tr>
<td>UV(_{CTRL})</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>V(_{CTRL})</td>
<td>6</td>
<td>0.21(^b)</td>
</tr>
<tr>
<td>UV(_{H1})</td>
<td>24</td>
<td>0</td>
</tr>
<tr>
<td>V(_{H1})</td>
<td>11</td>
<td>0.35(^b)</td>
</tr>
<tr>
<td>UV(_{H3})</td>
<td>24</td>
<td>0</td>
</tr>
<tr>
<td>V(_{H3})</td>
<td>11</td>
<td>0.27(^b)</td>
</tr>
<tr>
<td>UV(_{CTRL})</td>
<td>6</td>
<td>0.80(^a)</td>
</tr>
<tr>
<td>V(_{CTRL})</td>
<td>6</td>
<td>0.66(^b)</td>
</tr>
<tr>
<td>UV(_{H1})</td>
<td>24</td>
<td>0.86(^a)</td>
</tr>
<tr>
<td>V(_{H1})</td>
<td>11</td>
<td>0.69(^b)</td>
</tr>
<tr>
<td>UV(_{H3})</td>
<td>24</td>
<td>0.85(^b)</td>
</tr>
<tr>
<td>V(_{H3})</td>
<td>11</td>
<td>0.71(^b)</td>
</tr>
</tbody>
</table>

\(^a\),\(^b\),\(^c\),\(^d\) Across rows, superscripts identify significant differences (\(\alpha=0.05\)) within groups by DPI.

\(^\dagger\)Homologous HI assays were made using influenza virus isolates A/Swine/OH/511445/2007 \(\gamma\) H1N1 and A/Swine/Illinois/02907/2009 Cluster IV H3N2. Means for the HIs were based on results \(\geq 1:20\).

Vaccinated\(^e\) animals received vaccinations DPI -42 and -21.
Table 3. Summary of antibody assay results: count of antibody positive animals by vaccination status and IAV inoculant over time

<table>
<thead>
<tr>
<th>Assay by Treatment Group</th>
<th>No. of pigs</th>
<th>Day Post Inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>H1N1 HI (Pos ≥ 20)</td>
<td>UV_CTRL</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>V_CTRL</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>UV_H1</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>V_H1</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>UV_H3</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>V_H3</td>
<td>11</td>
</tr>
<tr>
<td>H3N2 HI (Pos ≥ 20)</td>
<td>UV_CTRL</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>V_CTRL</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>UV_H1</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>V_H1</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>UV_H3</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>V_H3</td>
<td>11</td>
</tr>
<tr>
<td>H1N1 Indirect ELISA a (Pos S/P ≥ 0.40)</td>
<td>UV_CTRL</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>V_CTRL</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>UV_H1</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>V_H1</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>UV_H3</td>
<td>24</td>
</tr>
</tbody>
</table>
|                          | V_H3        | 11 | 0  | 0  | 3  | 2  | 1  | 2  | 1
| H3N2 Indirect ELISA b (Pos S/P ≤ 0.40) | UV_CTRL | 6  | 0  | 0  | 0  | 0  | 0  | 0  |
|                          | V_CTRL      | 6  | 2  | 1  | 0  | 1  | 1  | 1  |
|                          | UV_H1       | 24 | 0  | 0  | 0  | 0  | 0  | 0  |
|                          | V_H1        | 11 | 5  | 6  | 6  | 3  | 4  | 5  | 1
|                          | UV_H3       | 24 | 0  | 0  | 2  | 1  | 2  | 1  | 2
|                          | V_H3        | 11 | 2  | 6  | 8  | 9  | 5  | 8  | 6
| NP blocking ELISA c (Pos S/N ≤ 0.60) | UV_CTRL | 6  | 0  | 0  | 1  | 0  | 0  | 0  |
|                          | V_CTRL      | 6  | 3  | 0  | 0  | 0  | 1  | 0  | 0
|                          | UV_H1       | 24 | 0  | 23 | 24 | 24 | 22 | 22 | 23
|                          | V_H1        | 11 | 3  | 11 | 11 | 10 | 10 | 10 | 9
|                          | UV_H3       | 24 | 0  | 21 | 23 | 23 | 23 | 24 | 23
|                          | V_H3        | 11 | 3  | 10 | 11 | 10 | 11 | 10 | 9

† (S/N = 0.598)
Vaccinated g animals received vaccinations DPI -42 and -21.
Table 4. Assay performance estimates based on receiver operating characteristic curve analyses

<table>
<thead>
<tr>
<th>Assay by sample set†</th>
<th>Total samples (negative, positive)</th>
<th>AUC (95% CI)</th>
<th>Designated cutoff (sensitivity, specificity)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1N1 HI (H1N1 data)</td>
<td>423 (279, 144)</td>
<td>1.00 (0.991, 1.00)</td>
<td>≥ 1:20 (100%, 100%)</td>
</tr>
<tr>
<td>H3N2 HI (H3N2 data)</td>
<td>423 (279, 144)</td>
<td>1.00 (0.991, 1.00)</td>
<td>≥ 1:20 (100%, 100%)</td>
</tr>
<tr>
<td>H1N1 HI (cumulative data)</td>
<td>567 (279, 288)</td>
<td>0.759 (0.721, 0.793)</td>
<td>≥ 1:20 (50.3%, 100%)</td>
</tr>
<tr>
<td>H3N2 HI (cumulative data)</td>
<td>567 (279, 288)</td>
<td>0.749 (0.711, 0.784)</td>
<td>≥ 1:20 (50.0%, 99.6%)</td>
</tr>
<tr>
<td>H1N1 ELISA&lt;sup&gt;a&lt;/sup&gt; (H1N1 data)</td>
<td>423 (279, 144)</td>
<td>0.936 (0.909, 0.958)</td>
<td>≥ 0.40 (1.4%, 100%)</td>
</tr>
<tr>
<td>H3N2 ELISA&lt;sup&gt;b&lt;/sup&gt; (H3N2 data)</td>
<td>423 (279, 144)</td>
<td>0.936 (0.908, 0.957)</td>
<td>≥ 0.40 (4.9%, 99.6%)</td>
</tr>
<tr>
<td>H1N1 ELISA&lt;sup&gt;a&lt;/sup&gt; (cumulative data)</td>
<td>567 (279, 288)</td>
<td>0.844 (0.812, 0.873)</td>
<td>≥ 0.40 (0.7%, 100%)</td>
</tr>
<tr>
<td>H3N2 ELISA&lt;sup&gt;b&lt;/sup&gt; (cumulative data)</td>
<td>567 (279, 288)</td>
<td>0.872 (0.841, 0.898)</td>
<td>≥ 0.40 (2.8%, 99.6%)</td>
</tr>
<tr>
<td>NP ELISA&lt;sup&gt;c&lt;/sup&gt; (H1N1 data)</td>
<td>423 (279, 144)</td>
<td>0.989 (0.974, 0.997)</td>
<td>≤ 0.60 (96.5%, 99.6%)</td>
</tr>
<tr>
<td>NP ELISA&lt;sup&gt;c&lt;/sup&gt; (H3N2 data)</td>
<td>423 (279, 144)</td>
<td>0.997 (0.985, 1.00)</td>
<td>≤ 0.60 (94.4%, 99.6%)</td>
</tr>
<tr>
<td>NP ELISA&lt;sup&gt;c&lt;/sup&gt; (cumulative data)</td>
<td>567 (279, 288)</td>
<td>0.993 (0.982, 0.998)</td>
<td>≤ 0.60 (95.5%, 99.6%)</td>
</tr>
</tbody>
</table>

Cumulative ROC analyses were based on test results from expected negative samples, i.e., unvaccinated and uninoculated pigs (n = 279), and expected positive samples, i.e., unvaccinated, IAV-inoculated pigs at DPI ≥ 7 (n = 288). Subtype (H1N1 or H3N2) ROC analyses used results from expected negatives (n = 279) and expected positives from unvaccinated pigs inoculated with either H1N1 or H3N2 viruses (n = 144).
Table 5. NP blocking ELISA\(^c\) LS means by DPI (95% CI) in vaccinated\(^g\) or unvaccinated pigs

<table>
<thead>
<tr>
<th>Status</th>
<th>Y = DPI + vax +DPI*vax(^f)</th>
<th>H1N1 or H3N2 inoculated (n = 70)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unvaccinated (n=48)</td>
<td>Vaccinated (n=22)</td>
</tr>
<tr>
<td>DPI -42(^*)</td>
<td>0.89</td>
<td>NA</td>
</tr>
<tr>
<td>DPI -21(^††)</td>
<td>0.89(^a)</td>
<td>0.88(^a)</td>
</tr>
<tr>
<td>DPI -7(^††)</td>
<td>0.88(^a)</td>
<td>0.76(^b)</td>
</tr>
<tr>
<td>DPI 0</td>
<td>0.85(^a)</td>
<td>0.69(^b)</td>
</tr>
<tr>
<td>DPI 7</td>
<td>0.43(^a)</td>
<td>0.25(^b)</td>
</tr>
<tr>
<td>DPI 14</td>
<td>0.38(^a)</td>
<td>0.26(^b)</td>
</tr>
<tr>
<td>DPI 21</td>
<td>0.36(^a)</td>
<td>0.30(^a)</td>
</tr>
<tr>
<td>DPI 28</td>
<td>0.34(^a)</td>
<td>0.32(^a)</td>
</tr>
<tr>
<td>DPI 35</td>
<td>0.35(^a)</td>
<td>0.32(^a)</td>
</tr>
<tr>
<td>DPI 42</td>
<td>0.33(^a)</td>
<td>0.44(^b)</td>
</tr>
</tbody>
</table>

\(^f\) Tukey Kramer test of multiple comparisons was used to assess differences between multifactorial variables in the full model: [DPI|inoculum|vaccine status].

\(^a,b\) Superscripts identify statistically significant differences (\(\alpha = 0.05\)) between unvaccinated and vaccinated pigs by DPI.

\(^*\) DPI -42 included all 82 pigs prior to vaccination

\(^††\) DPI -21 and -7 included all 82 pigs, 54 unvaccinated, 28 vaccinated.

Vaccinated\(^g\) animals received vaccinations DPI -42 and -21.
Figure 1. Cumulative distribution of NP blocking ELISA test results from expected negative samples (n = 279) and expected positive samples (n = 288).
GENERAL CONCLUSIONS

U.S. pork exports have grown over the last two decades, especially in the last 10 years (Table 1). In 1990, exports represented less than 1.6% of U.S. pork production (Brester et al., 1997). By 2012, exports had risen to 27% and were valued at $6.3 billion (USD) (http://www.agrimarketing.com/s/80406). As U.S. pork producers have become increasingly dependent upon exports, they also become more vulnerable to forces that affect export markets. This was poignantly illustrated in the 2009 H1N1 influenza pandemic when the closure of export markets caused a $1.3 billion loss to the U.S. pork industry between April 24 to December 31, 2009 (Pappaioanou and Gramer, 2010). Foreign animal diseases pose a threat to the industry, as well. For example, Rendleman and Spinelli (1999) estimated the cost of an African swine fever virus outbreak in the U.S. at $4,500,000,000 to $5,445,000,000 in 1992 dollars. While appropriate for the industry for that time, this estimate does not account for the current volume of exports and the loss of these markets.

Table 1. Total U.S. pork exports (2003-2012)*

<table>
<thead>
<tr>
<th>Year</th>
<th>Metric Tons Exported</th>
<th>Value ($USD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2012</td>
<td>2,262,109</td>
<td>$6,322,000,000</td>
</tr>
<tr>
<td>2011</td>
<td>2,255,334</td>
<td>$6,108,000,000</td>
</tr>
<tr>
<td>2010</td>
<td>1,917,649</td>
<td>$4,781,000,000</td>
</tr>
<tr>
<td>2009</td>
<td>1,865,745</td>
<td>$4,329,000,000</td>
</tr>
<tr>
<td>2008</td>
<td>2,052,447</td>
<td>$4,884,000,000</td>
</tr>
<tr>
<td>2007</td>
<td>1,305,622</td>
<td>$3,154,000,000</td>
</tr>
<tr>
<td>2006</td>
<td>1,262,499</td>
<td>$2,864,000,000</td>
</tr>
<tr>
<td>2005</td>
<td>1,157,689</td>
<td>$2,634,000,000</td>
</tr>
<tr>
<td>2004</td>
<td>1,023,413</td>
<td>$2,227,000,000</td>
</tr>
<tr>
<td>2003</td>
<td>757,406</td>
<td>$1,582,000,000</td>
</tr>
</tbody>
</table>

Given the value of the industry to the U.S. economy, it would seem prudent to proactively protect this resource by creating the technical, logistic, and analytical capacity to efficiently collect and respond to data on endemic and/or exotic infectious diseases in the U.S. swine herd. If a surveillance system were to meet this objective, it must use on-farm, real-time, infectious disease data collected in all phases of swine production. With testing performed in NAHLN laboratories, electronic interconnectivity among laboratories and USDA would provide for real-time sharing and streaming of diagnostic data for surveillance.

The goal of the research described in this dissertation was to (1) describe the fundamental parameters critical to a successful surveillance program given the significant evolution of the U.S. swine industry over the last 20 years, and then (2) evaluate the technical and diagnostic challenges associated with on-farm ante mortem sampling using influenza A virus infection in pigs as a model. In particular, diagnostic evaluation of oral fluid specimens was included because of its potential to fulfill the requirement for an easily collected, specimen.

A fundamental problem identified through this work was a deficiency in the reproducibility of results among diagnostic laboratories (Chapter 2). This outcome was unexpected given that collaborating laboratories were considered expert laboratories in swine diagnostics and included participants in the U.S. National Animal Health Laboratory Network (NAHLN). Although the ring test focused on the evaluation of influenza A virus nucleic acid assays, it brings into question the reproducibility of other tests commonly used in swine diagnostics. This issue is significant because surveillance without reproducible (between laboratory) and repeatable (within laboratory) tests is untenable.

Specific to influenza A virus, oral fluid was a superior surveillance specimen when tested by real-time, reverse transcription polymerase chain reaction, whereas nasal swabs were preferable for virus isolation (Chapter 3). Likewise, a current commercial nucleoprotein antibody ELISA was found to be an excellent serologic screening tool for influenza A virus exposure (Chapter 4). Recent work has also demonstrated success with detection of influenza A virus nucleoprotein antibody in oral fluid (Panyasing et al., 2012, 2013).
The use of oral fluid diagnostic specimens for monitoring herd health is becoming commonplace among U.S. swine veterinarians. Amenable to testing by antibody-, nucleic acid- or antigen capture-based assays, there is no easier method of collecting infectious disease information than analysis of oral fluids. On the farm, integration of surveillance data with herd records would provide the means to (1) identify the circulation of specific pathogens; (2) quantify their effects on pig health and productivity; (3) target interventions to the correct pathogen and population; and (5) time the intervention for maximum effect. At the regional level, oral fluid-based surveillance would make producer-driven area control programs more practical and affordable.

It is imperative that research on the diagnostic applications of oral fluids continue. If future research confirms past experience, oral fluid-based diagnostic tests for foreign animal diseases could be developed and should be performed routinely in NAHLN laboratories in order to provide immediate capacity for high-volume testing in the event of an emergency. Likewise, a surveillance infrastructure based on assays optimized for oral fluids would facilitate rapid collection of data for national control-and elimination-programs or containment of foreign animal disease agents. Achieving this vision will provide the means to safeguard the economic viability of U.S. pig producers.
REFERENCES


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