Diagnosis of swine influenza

Bruce H. Janke, DVM, PhD

Summary

In recent years, swine influenza has become recognized as a significant contributor to the porcine respiratory disease complex (PRDC), which is causing severe problems in many swine operations. Infection with swine influenza virus (SIV) is not always clinically evident and diagnostic tests often must be conducted to detect the presence of the virus, especially in cases of enzootic infection. A variety of assays are available to detect SIV or the antibody induced by the virus. The appearance of antigenically variant H1N1 strains and, more recently, of new H3N2 subtype strains in swine populations in the United States has raised questions about cross-protection and about our ability to detect the new strains with currently available diagnostic tests. In this review, we present information to update the reader on the methods available to detect swine influenza, to differentiate among SIV strains, and on the optimum use of these tests.

Keywords: swine, swine influenza virus, diagnosis, H3N2 subtype

Received: October 20, 1999
Accepted: January 13, 2000

Historically, swine influenza has been considered relatively easy to diagnose. In its classical epizootic form, which induces a harsh barking cough and high fever, the infection could be identified on the basis of clinical signs alone. Because the disease passed through herds very quickly with only short-lived effects, the infection was not considered a significant problem and treatment was usually deemed unnecessary.[1] Recently, an enzootic form of the disease has appeared in large operations as a part of the porcine respiratory disease complex (PRDC).[2] The ubiquitous presence of the virus and the significant accompanying losses incurred due to respiratory disease has increased the importance of infections. Perhaps this is best highlighted by the demand for a vaccine.

The enzootic form of infection is not clinically dramatic or unique, and diagnostic testing is often necessary to detect infection. To further complicate matters, new strains of swine influenza virus (SIV) have appeared in swine populations in the United States and Canada during the last decade, derived through the well-defined processes of antigenic drift and shift which have been described for influenza viruses that affect other species. Antigenically variant or atypical swine influenza viruses were identified first in Canada and later in the United States in the late 1980s and early 1990s.[3,4] Within the past year, an even more dramatic and important change has occurred in the United States: a new subtype (H3N2) was identified as a significant pathogen in swine. This is the first occurrence of a new subtype of SIV in the United States since 1918.[5]

Some of the most dramatic epizootics of H3N2 infections observed recently occurred in vaccinated pregnant gilts and sows that subsequently aborted in high numbers. However, abortion appears to result from the high fevers induced by infection of naive dams rather than through direct infection of the fetus. There is one report of isolation of virus directly from a single porcine fetus from a sow that aborted during an outbreak,[6] but previous research on H1N1 SIV has indicated that the virus does not leave the respiratory tract to any great extent and usually will not be found in aborted fetuses.[7] Similar studies have not yet been conducted with H3N2 virus, but at this time, there is no reason to believe this subtype will act any differently. Once an animal has recovered and become immune, the virus is cleared from that animal (i.e., no carrier state has been identified); thus, sows that abort should not have subsequent reproductive problems. Ongoing reproductive problems reported in herds that suffered through an abortion epizootic of H3N2 SIV infection may have been due to incomplete herd immunity with subsequent abortions occurring in gilts or sows that did not become infected during the initial epizootic.

Reports of atypical H1N1 SIV led to concerns about our ability to detect variant strains with the diagnostic tests already in place and whether the amount of cross-protection afforded pigs that were vaccinated or had been infected with classical strains was adequate. Dramatic epizootics due to infection with the new H3N2 strains have occurred, even in herds heavily immunized with a vaccine considered efficacious against the H1N1 strains. Apparently, these vaccines offer little cross-protection against infection with H3N2 strains. This lack of cross-reaction has renewed concerns about our ability to detect both H3N2 and H1N1 infections with the current antibody-dependent techniques.

In this manuscript, we present information to update the reader on the methods available to detect swine influenza and to differentiate among SIV strains, and provide guidance for the optimum use of these tests.

Swine influenza virus

Influenza viruses are double-stranded RNA viruses that belong to the family Orthomyxoviridae. The viruses are classified into types A, B, and C according to the composition of nucleoproteins and matrix proteins. The nucleoproteins support the nucleic acids of the viral genome, and the matrix proteins line the inside of the viral envelope.[8] These proteins are relatively less important for protective immunity when compared to the external proteins. They are involved in cell-mediated immunity and recovery from infection. These

Veterinary Diagnostic Laboratory, Iowa State University, Ames, Iowa 50011

This diagnostic note has been peer refereed.

This article is available online at `http://www.aasp.org/shap.html`.

Janke BH. Diagnosis of swine influenza. *Swine Health Prod.* 2000;8(2):79–84.

proteins are relatively conserved; i.e., they show only minimal variation among strains, and thus detection tests aimed at finding these proteins will identify all of the strains with which we are concerned. Nearly all swine influenza viruses worldwide are type A viruses; only a few isolated reports from Asia have indicated infection of swine with influenza viruses of other types.

The external proteins that project from the surface of the virus—the hemagglutinin (HA) and the neuraminidase (NA)—are more significant for infection and immunity. The HA is responsible for attachment of the virus to the host receptor and infection of the host cell. This protein is also the major antigen against which the host raises an antibody response. The NA protein functions in release of progeny virions from the infected host cell, and helps reduce self-agglutination of the virus. This protein has a less prominent role as an immunogen. Fifteen HA proteins and nine NA proteins have been identified in influenza viruses in animals, birds, and humans. The degree of homology in the amino acid sequence between HA proteins of different subtypes varies from 25%–80%, while homology between HA proteins within the same subtype is generally greater than 90%.[9] Only H1 and H3 hemagglutinins have been recovered from swine worldwide.

The H3N2 virus that appeared in North Carolina swine last year appeared to be a reassortant virus that contained the genes for all but one of the internal proteins derived from the pre-existing H1N1 swine virus. The genes for the HA and NA proteins and one internal protein, PB1, are thought to have been derived from a recent human H3N2 strain.[10] This event in itself is somewhat unusual in that most infections of swine with H3N2 strains in other parts of the world have been incidences of human strains entering swine populations intact or by reassortants of human and avian strains.[8,11] Of interest, and perhaps concern, is the fact that the H3N2 viruses from different parts of the United States that have been studied to date differ in composition. Like the original North Carolina isolate, three H3N2 viruses isolated from swine in the Midwestern United States (Iowa, Minnesota, and Texas) contain similar HA, NA, and PB1 genes from a human strain and three of the internal genes from the classical swine H1N1 virus. However, the Midwest isolates also contain two internal genes, PB2 and PA, that appear to have been derived from an avian strain.[10] The HA of the original North Carolina isolate is more divergent from the more closely related Midwest isolates. The HA1 portion of the North Carolina isolate differs from the three Midwest isolates in 18–20 amino acids. The Midwest isolates differ from each other in only two to four amino acids.

The significance of these differences is not clear, but the fact that the few strains studied so far do not appear to be the same is intriguing. A shift in subtype had not occurred in the 75 years that have passed since the disease was first identified in the United States. When a new subtype did appear, it seemed to spread very rapidly throughout the country. Were there multiple incidences of co-infection and reassortment in separate swine populations, or did additional changes occur as the new subtype spread through naive populations to produce this variation? Retrospective analysis of the composition of multiple isolates from different regions of the country and from different times is expected to provide more information.

In general, the H1 and H3 hemagglutinins are the hemagglutinins that are most divergent from each other, sharing only approximately 25% homology.[9] Thus, very little cross-reactivity between antibodies for these proteins would be expected, which could affect cross-protection in the field and antibody-based diagnostic assays. Infection or vaccination against H1N1 strains would likely provide very little protection against infection with H3N2. This appeared to be the case in the vaccinated sows that were so severely affected during the H3N2 epizootics in late 1998 and early 1999. Most of the SIV detection tests used in veterinary diagnostic laboratories use antibody directed against the conserved type-specific antigens and so have been successful in detecting both H1N1 and H3N2 subtypes. However, additional differential serologic tests have had to be developed to detect seroconversion to the H3N2 strains.

The HA amino acid sequences in the H1 and H3 subtypes is sufficiently different that cross-reacting antibodies would not be expected, but the cross-reactivity between strains within the same subtype is less predictable from assessment of amino acid sequence homology alone. Variations in HA1 amino acid sequences between typical and atypical H1N1 strains were never more than 15–18 amino acids, resulting in homologies of 94%–96%.[4,12] These minor variations did not appear to have any affect on cross-protection or to affect diagnostic test sensitivity.[13] However, some researchers who are well-acquainted with influenza, extrapolating from experiences with H3 influenza viruses in other species, have predicted that the H3 hemagglutinin in H3N2 strains will be less stable than the H1 and that greater antigenic drift may occur as the virus continues to move through swine populations.

How much drift is necessary before it has clinical and diagnostic significance? The answer is unclear and may be affected more by the location of changes on the protein than by the total sequence change. Comparative serologic studies on the cross-reactivity of strains may provide more clinically relevant clues than nucleic acid and amino acid sequence studies. A network of collaborating centers of influenza research established by the World Health Organization to address international surveillance of influenza activity annually examines information on currently circulating human strains to advise on the appropriate strains for inclusion in human influenza vaccines to be produced in the following year.[14] Serologic data are used in these discussions.

Studies comparing the amino acid sequence of the HA from H3N2 isolates from Iowa, North Carolina, Minnesota, Texas, and Illinois have indicated that these strains are 95%–98% homologous.[10,15] From past studies with H1N1 strains,[13] this degree of homology might not be expected to greatly affect cross-protection, unless the change at any particular point(s) in the amino acid sequence markedly affected protein conformation. Such a critical point mutation may have occurred because antiserum raised in caesarian-derived, colostrum-deprived (CDCD) pigs against the original North Carolina strain does not react with the Midwest strains in hemagglutination inhibition tests. Antisera against the Midwest strains also does not react with the North Carolina strain.[16] Other isolates resembling the Midwest isolates have subsequently been recovered from swine in North Carolina, but no additional isolates similar to the initial North

Carolina strain have been found in that state.[17] Whether strains resembling the original North Carolina isolate are present elsewhere in the country has not yet been determined.

Diagnostic tests that detect virus, viral proteins (antigens), or viral nucleic acid

Fluorescent antibody (FA) test

This test uses either antiserum prepared against whole virus (polyclonal antibody) or an antibody against a specific protein (monoclonal antibody), which is usually applied to frozen sections of lung from pigs submitted for laboratory examination. The reagent used in the FA test in most diagnostic laboratories is a polyclonal antiserum offered by National Veterinary Services Laboratories, which was prepared by injection of pigs with an H1N1 strain of SIV initially isolated from swine in 1973 (A/Sw/Ia/73).

The animals from which the antibody was harvested were exposed to whole virus, and antibody against both internal and external proteins are in the antiserum. The reagent will detect both H1N1 and H3N2 strains, although some labs have reported that fluorescence in lungs infected with H3N2 virus is less intense. This test can be completed within several hours and results can be available the same day if samples are submitted early enough in the day. Fresh tissue should be chilled but not frozen.

Immunohistochemical (IHC) test

This test is similar in principle to the FA test but usually refers to the use of antibody applied to sections of formalin-fixed tissue. The sensitivities of the FA test and IHC test are similar,[18] but the advantage of the IHC test is that it can be applied to tissues optimally fixed at the time of necropsy without loss of tissue integrity during transport to the laboratory. To reduce background staining, monoclonal antibodies are usually used. The antibodies used at the Veterinary Diagnostic Laboratory at Iowa State University (ISU VDL) are directed against type A nucleoproteins and thus will detect both H1N1 and H3N2 strains. We are currently working to develop a differential IHC test that will allow us to determine whether infection was with H1N1 or H3N2 strains, eliminating the need to isolate virus. This test requires processing of tissues into histopathologic sections, but results can be available the next day if formalin-fixed tissues are submitted.

Antigen-capture ELISA

A commercial ELISA test (Directogen™ ; Becton Dickinson Microbiology Systems; Sparks, Maryland) developed for detection of influenza virus in throat or nasal swabs in humans is also used in some veterinary laboratories. This test will detect both H1N1 and H3N2 strains but will not differentiate between subtypes. Studies on experimentally inoculated pigs suggest that the ELISA is only slightly less sensitive than egg inoculation in detecting virus shedding.[19] In practice, however, the test has not proven to be as sensitive as virus isolation when applied to nasal swabs, and excess mucus or blood in the sample can interfere with the test. The ELISA test has been successfully used on swabs of small airways taken directly from lungs of pigs at necropsy. Care should be taken to swab airways that lead from affected lobules. Like FA tests, the ELISA tests can be conducted relatively quickly, but they are considerably more expensive. Freezing of samples may reduce the sensitivity of this test.

Polymerase chain reaction (PCR) tests

Nucleic acid primers have been developed in several research laboratories, including the ISU VDL. These primers will hybridize with and detect both H1N1 and H3N2 viruses and differentiate between them. These probes have been used primarily to characterize virus isolates. These probes could also be used directly on clinical specimens without the necessity of isolation. The sensitivity of such use has not yet been established.

In a preliminary study, primers against the conserved internal nucleoprotein detected 100% of the infected lung tissues when compared to virus isolation.[20] Subtype-specific primers used similarly in a multiplex PCR assay detected about 75% of the infections. Information is not yet available on efficacy for testing nasal swabs.

The reagents are more expensive and the procedures more labor-intensive and time-consuming than with other tests. Test procedures require 2–3 days for completion.

Virus isolation

Historically, virus isolation of influenza viruses from most host species has been conducted in embryonated chicken eggs. Because of the expense of maintaining a continuous egg supply and the labor-intensive procedures, not all laboratories have made this diagnostic test routinely available. Isolation can be conducted on nasal secretions and lung homogenate but the virus is not particularly hardy and samples must be kept cool and moist. Secondary bacterial infection also can interfere with virus isolation procedures by killing the chick embryo before viral multiplication can occur. Virus growth in eggs is determined by detecting hemagglutinating activity in egg fluids 5 days after inoculation. Determining that the hemagglutination activity is due to influenza virus infection and identifying subtype is then conducted on egg allantoic fluids by reaction with antisera or through PCR. These procedures take more time than other tests and are better suited for characterizing virus than for diagnosis. Cell cultures (MDCK cells or primary porcine kidney cells) also are being used for isolation of influenza viruses in some laboratories. When optimized, this method has been reported to be as sensitive as egg inoculation.[17] Virus isolation procedures take longer than other detection tests, and success is dependent on the amount of virus present and quality of the sample. With either method, allow 1–2 weeks for isolation and characterization.

Diagnostic tests that detect circulating antibody to SIV (serology)

Hemagglutination inhibition test

The classic serologic test for detecting antibody against SIV is the hemagglutination inhibition (HAI or HI) test. This test detects circulating antibody that binds to the HA protein on the surface of the virus, thus preventing the virus particles from attaching to the surface of erythrocytes to form a virus-erythrocyte meshwork (hemagglutination). The test is relatively simple and can be completed within a few hours. Serum to be tested is mixed with virus of known concentration and time is allowed for any antibody present to react with this virus. Then the indicator reagent (rooster or turkey erythrocytes) is added to determine whether the virus in the test is still unbound and can agglutinate the

erythrocytes. The amount of antibody present in the serum is determined by running serial two-fold dilutions of the serum against the same concentration of virus. The titer is the dilution at which there is no longer sufficient antibody present to prevent or inhibit hemagglutination.

The ability of the test to detect antibody against a particular strain of virus in the field depends on the antigenic relationship between the field virus strain that induced antibody and the virus strain used in the test. Veterinary diagnostic laboratories in the United States have used classic type A subtype H1N1 virus in developing and running their routine SIV HI serology tests because that was the only virus of concern. The virus used in this test by most veterinary laboratories in the United States is the A/Sw/Ia/73 (H1N1), supplied by National Veterinary Services Laboratories. With the identification of antigenically variant type A subtype H1N1 strains, there was concern that these standard tests would not be able to detect antibody against these variants. Studies were conducted at the ISU VDL comparing HI test results with antibody induced in pigs with classical and antigenically variant H1N1 strains against homologous and heterologous strains used in the test. Results indicated that the degree of antigenic variation in the HA proteins between classic or typical H1N1 strains and atypical or antigenically variant H1N1 strains did not affect the ability of the test to detect antibody against the other H1N1 strains. Sensitivity of the test was not reduced.[13] Unexpectedly, similar studies with the original North Carolina H3N2 isolate and the Midwest H3N2 isolates have indicated that antibody induced by the North Carolina virus does not cross react with the Midwest strains and vice versa. [16]

The HI test also is considered a relatively sensitive test as the HA protein is quite antigenic and stimulates high circulating antibody concentrations. Titers of 1:40 or less may include nonspecific reactions; titers of 1:80 and above are considered positive and specific. Antibody can be detected within 5–7 days of infection with many pigs exhibiting titers reaching 1:80 by 1 week postinfection (PI) and peaking at 1:320–1:640 by 2–3 weeks PI. Antibody concentrations will remain high for several weeks before beginning to decline (Tables 1 and 2). Passive antibody in pigs in infected herds will disappear in most pigs by 6 weeks of age. Vaccination of sows prefarrowing will prolong this passive antibody until about 16 weeks of age (Table 3).[21]

There is very little cross-reaction between the HA proteins of H1N1 and H3N2 subtype strains of SIV, and thus the standard HI test using the H1N1 strains will not detect antibody against H3N2 strains. Some difficulties have been encountered in developing a similar HI test simply by using the H3N2 virus in the test. More manipulation of the reagents has been necessary, but a differential test is now available. Laboratories in the Midwest that have developed the test have been using a Midwest (Texas or Iowa) strain. At this point, studies suggest that the test does not have

Table 1

	Hemagglutination inhibition titer				
Days postinoculation	20	40	80	160	320
3	5	0	0	0	0
7	0	2	2	1	0
14	0	2	2	0	1
21	0	1	2	2	0

Table 2

	Days Postinoculation			
Pig#	0	7	14	28
1	0/0	80/160	320	160/320
2	0/0	160	640	320
3	0/0	160	160/320	160
4	0/0	40/80	320	ND
5	0/0	80	320	160
6	0/0	160	320	320
7	0/0	80/160	80/160	160
8	0/0	20/40	160	160
9	0/0	320	320	320
10	0/0	160	320	320

Table 3

Sow vaccination status	Age (wks)	No. of pigs	No. of pigs with each reciprocal of the hemagglutination inhibition titer							
			>10	10	20	40	80	160	320	640
Yes	0	23	0	0	0	0	0	0	0	23
No	0	15	0	0	2	1	1	4	0	7
Yes	2	23	0	0	0	0	0	1	5	7
No	2	15	1	1	2	3	4	3	1	0
Yes	4	23	0	0	0	0	1	7	4	11
No	4	15	3	2	2	5	3	0	0	0
Yes	6	23	0	0	1	5	3	9	5	17
No	6	15	5	5	4	1	0	0	0	0
Yes	8	23	1	0	6	6	3	7	0	0
No	8	15	14	1	0	0	0	0	0	0
Yes	12	23	5	6	2	6	4	0	0	0
No	12	15	15	0	0	0	0	0	0	0
Yes	16	23	17	4	2	0	0	0	0	0
No	16	15	15	0	0	0	0	0	0	0
Yes	20	18	18	0	0	0	0	0	0	0
No	20	15	14	0	0	0	0	0	0	0

cross-reactivity problems with H1N1 strains and will detect antibody against any of the Midwest strains. However, the test will not detect antibody induced by the original North Carolina strain. Because heat treatment of the serum is conducted before use in the H1N1 test and because such treatment will interfere with its use in the H3N2 test, you should decide whether to test for one or both viruses at the time of submission. Serum tested for antibody against H3N2 can be subsequently tested for antibody against H1N1 but not vice versa.

Enzyme-linked immunosorbent assay for antibody

An ELISA test for SIV antibody has been previously developed in a few research laboratories, but studies comparing the results of this test with the H1 test indicated poor correlation, and the test has not been used routinely in most veterinary diagnostic laboratories. More recently, development of an SIV ELISA has been undertaken by the company that successfully produced such tests for PRV and PRRSV, and this test may be commercially available soon.[22, 23] Such tests offering differential screening of H1N1 and H3N2 antibody would be very beneficial.

Samples for diagnosis of SIV infection

Nasal swabs

Nasal swabs from acutely affected pigs can be used for virus isolation. You should select pigs with high fevers and clear nasal discharge for such samples. Most pigs will shed virus for 5–7 days after infection. Swabs with synthetic fiber (rayon or dacron) tips should be used as cotton will inactivate the virus. Swabs also should be kept moist and cool to prevent desiccation and inactivation of the virus. Culturettes™ (Baxter Healthcare Corporation, Deerfield, Illinois) with crush bulbs to keep the tips moist work well. Other swabs can be broken off into small vials or snap cap tubes with physiologic saline or cell culture media. Freezing may have a slight negative effect on use of these swabs for virus isolation and should only be done if swabs cannot be delivered to the laboratory in a timely manner.

Nasal swabs also can be used to collect samples for use in the antigen-detection ELISA kits. Excess mucus or blood on the swabs can interfere with successful use of the test. One lab has reported that this test has not worked as well on nasal swabs as on swabs directly applied to small airways in lung tissue. PCR diagnostic tests also have been applied to nasal swab samples but the relative sensitivity of this analysis versus VI and ELISA has not been evaluated. Freezing also may adversely affect the use of swabs in the ELISA test.

Lung tissue

Fresh and fixed lung collected at necropsy from pigs with respiratory disease are the most commonly used samples for diagnosis. Swine influenza virus initially infects the epithelium lining the airways, and the resulting lesion is predominantly a bronchopneumonia characterized by multiple coalescing foci of lobular consolidation in cranioventral portions of lung. These areas should be submitted for diagnostic evaluation. Fresh tissue (chilled, not frozen) can be used for FA test and VI studies, and formalin-fixed tissue for IHC test and histopathologic examination. Experimental studies have indicated that peak virus load in the airways is present at 24 hours after infection even before gross lesions develop.[24] Virus usually can still be detected in bronchioles and alveoli at 48–72 hours after infection. In many pigs, very little virus may be found by FA or IHC by 72 hours PI, and distribution is often quite focal.

Histopathologic examination can demonstrate lesions suggestive of SIV infection for about 2 weeks after infection. By 3 weeks PI, recovery is almost complete. The later that lungs are examined after infection, the more difficult the lesion becomes to evaluate. Porcine circovirus can induce bronchiolar damage similar to that induced by SIV and both *Mycoplasma hyopneumoniae* and SIV infections induce significant peribronchiolar and perivascular lymphocytic cuffing. Samples from more than one pig are recommended to address the diagnostic difficulties imposed by the focal nature of the lesions and the pig-to-pig variation in timing of infection, especially in cases of enzootic respiratory disease.

Serum samples

Serum samples for serology studies should be collected at least a week after infection is suspected to have occurred. Most pigs will have titers of at least 1:80 at a week after infection and high titers (1:320–1:640) should be expected in at least some of the pigs sampled 14–21 days after the group was infected. Paired samples may be necessary in vaccinated herds.

Abortion

Direct isolation of virus from nasal swabs of sows that are acutely ill or performing serologic studies on affected groups are the preferred methods to determine whether SIV is involved in reproductive problems. Attempts to isolate SIV from fetuses is likely to be unrewarding.

Acknowledgements

I would like to thank the following people for providing information for this report: K-J Yoon (Iowa State University); S Swenson (National Veterinary Service Laboratory); J Collins, S Goyal, K Rossow (University of Minnesota); D Johnson, P Sweet, L Fawcett (South Dakota State University), C Olsen (University of Wisconsin), G Erickson (University of North Carolina), G Stevenson (Purdue University), R Sibbel (Schering-Plough).

References

1. Easterday BC, Hinshaw VS. Swine influenza. In: *Diseases of Swine*. Leman AD, et al.(eds), 7th ed., Iowa State University Press, Ames, Iowa. 1992;349–357.

2. Janke BH. Classic swine influenza. *Large Anim Prac.* 1998;19:24–29.

3. Dea S, Bilodeau R, Sauvageau C, et al. Antigenic variant of swine influenza virus causing proliferative and necrotizing pneumonia in pigs. *J Vet Diagn Invest.* 1992;4:380-392.

4. Olsen CW, McGregor MW, Cooley J, et al. Antigenic and genetic analysis of a recently isolated H1N1 swine influenza virus. *Am J Vet Res.* 1993;54:1630-1636.

6. Madec F, Kaiser C, Gourreau JM, et al. Consequences pathologiques d'un episode grippal severe (virus swine A/H1N1) dans les conditions naturelles chez la truie non immune en debut de gestation. *Comp Immun Microbiol Infect Dis*. 1989;12:17–27.

7. Brown TT, Mengeling WL, Pirtle EE. Failure of swine influenza virus to cause transplacental infection of porcine fetuses. *Am J Vet Res*. 1982;43:817–819.

8. Webster RG, Bean WJ, Gorman OT, et al. Evolution and ecology of influenza A viruses. *Microbiol Rev.* 1992;56:152-179.

9. Murphy BR, Webster RG. Orthomyxoviruses. In: *Virology.* Fields BN, Knipe DM, et al. (eds), Raven Press, Ltd, New York. 1990;1091-1152.

10. Zhou NN, Senne DA, Landgraf JS, Swenson SL, Erickson G, Rossow K, Liu L, Yoon K-J, Krauss S, Webster RG. Genetic reassortment of avian, swine and human influenza A viruses in American pigs. *J Virol.* 1999;73:8851–8856.

11. Castrucci MR, Donatelli I, Sidoli L, et al. Genetic reassortment between avian and human influenza A viruses in Italian pigs. *Virol.* 1993;193:503–506.

12. Rekik MR, Arora DJS, Dea S. Genetic variation in swine influenza virus A isolate associated with proliferative and necrotizing pneumonia in pigs. *J Clin Microbiol.* 1994;32:515–518.

14. Watson JM. Surveillance of influenza. In: *Textbook of Influenza*. Nicholson KG, Webster RG, Hay AJ (eds), Blackwell Science Ltd., Oxford. 1998;207–216.

18. Vincent LL, Janke BH, Paul PS, Halbur PG. A monoclonal-antibody-based immunohistochemical method for the detection of swine influenza virus in formalin-fixed, paraffin-embedded tissues. *J Vet Diagn Invest.*1997;9:191–195.

References-nonrefereed

5. Janke BH, Yoon KJ, Halbur PG, Sorden SD, Senne DA, Swenson SL, Schmitt B. Isolation of a new subtype (H3N2) of swine influenza virus from pigs with respiratory disease in the north central U.S. *Proc IX Internatl Symp World Assoc Vet Lab Diagn*. 1999:51.

13. Janke BH. Significance of antigenic variation in swine influenza virus for serodiagnosis. *Proc Sw Dis Conf Pract*. 1997:7–14.

15. Junker DE, Weston CQ, Valencia DM, et al. Molecular characterization of the hemagglutinin and neuraminidase genes from an H3N2 swine influenza virus isolate recovered from the U.S. *Proc Leman Sw Conf*. 1999;26(Suppl):5.

16. Swenson SL, et al. Experimental infection of pigs with H3N2 swine influenza virus. *Proc AAVLD*. 1999:42.

17. Erickson G, et al. New approaches to swine influenza virus isolation. *Proc AAVLD*. 1999:45

19. Swenson S, et al. A comparison of two enzyme immunoassays with egg inoculation for the detection of swine influenza virus from nasal swabs and lungs. *Proc AASP*. 1996;97

20. Harmon K, Krauss S, Yoon KJ. Development of a PCR based differential test for H1N1 and H3N2 swine influenza viruses. *Proc AAVLD*. 1999:44.

21. Thacker BJ, Cain CM, Janke BH, Kinker DR. Swine influenza virus passive antibody levels in pigs from vaccinated or nonvaccinated sows. *Sw Res Reports*. ISU, 1998.

22. Velek K, Hogan S, Liauw H. Development of an ELISA for the detection of antibodies to swine influenza virus in swine sera. *Proc AAVLD*. 1999;105.

23. Cornaglia E, Laganiere G, Lallier R, et al. A new competitive ELISA for serological monitoring of swine influenza. *Proc AAVLD*. 1999:41

24. Janke BH, et al. *Proc IX Internatl Symp World Assoc Vet Lab Diagn*. 1999:52