Peer reviewed

Brief communication

Enzyme-linked immunosorbent assay detection of antibodies against swine influenza virus in western Romania

Corina Pascu, DVM, PhD; Luminita Costinar, DVM, MSc, PhD; Viorel Herman, DVM, PhD

Summary

A total of 3651 blood samples collected from nonvaccinated pigs in 45 Romanian herds and tested by enzyme-linked immunosorbent assay against H1N1 and H3N2 subtypes revealed an H1N1 subtype seroprevalence of 44.4%. Prevalence differed by parity ($P < .01$), with the highest prevalence at parity 5-6.

Keywords: swine, influenza virus, seroprevalence

Received: February 5, 2011
Accepted: September 28, 2011

Resumen - Detección de anticuerpos contra el virus de influenza en el oeste de Rumania mediante el ensayo inmunoenzimático ligado a enzimas

Un total de 3651 muestras de sangre recolectadas de cerdos no vacunados en 45 piaras Rumanas y probadas con el ensayo inmunoenzimático ligado a enzimas contra los subtipos H1N1 y H3N2 revelaron una seroprevalencia del 44.4% del subtipo H1N1. La prevalencia difirió por paridad ($P < .01$), con la mayor prevalencia en la paridad 5-6.

Résumé - Détection d'anticorps contre le virus de l'influenza porcin en Roumanie occidentale à l'aide d'une épreuve immuno-enzymatique

Les tests effectués sur un total de 3651 échantillons sanguins prélevés de porcs non-vaccinés dans 45 troupeaux roumains à l'aide d'une épreuve immuno-enzymatique détectant les sous-types H1N1 et H3N2 ont révélé une séro-prévalence du sous-type H1N1 de 44.4%. La prévalence différait en fonction de la parité ($P < .01$), la prévalence la plus élevée était observée à la parité 5-6.

Swine influenza, a respiratory disease that affects pigs of all ages, is caused by type A influenza virus.[1] Morbidity rates can reach 100%, while mortality rates are generally low. Subtypes of swine influenza virus (SIV) most frequently identified in pigs include classical and avian H1N1, reassortant (r) H3N2, and rH1N2. In Europe, three main influenza A subtypes (H1N1, H1N2, H3N2) circulate in swine populations.[2] The H1N1 and H3N2 subtypes have been enzootic in several swine-producing countries in Europe for more than 20 years.[3-5] Surveillance and serological monitoring of influenza in swine populations is essential for adequate control and diagnosis of infection.[4] Swine influenza viruses are infectious to people as zoonotic agents. Zoonotic infections have been documented in the United States, Europe, New Zealand, and Hong Kong, in some cases resulting in the death of the people infected.[6]

The dramatic decrease in pig numbers in Romania during 1990-2002 is related to changes in the ownership of farms after the December 1989 revolution. Presently, approximately 5,793,000 domestic pigs were produced in 275 commercial farms, comparable to production in industrialized farming systems in other European Union member countries, with the highest number of pigs in west and northwest Romania.[7,8]

There have been few studies in Romania regarding SIV infection in swine herds. The aim of the present study was to assess the seroprevalence of swine influenza subtypes among domestic pigs of different ages raised in large farms located in densely swine-populated areas in western Romania.

Materials and methods

All samples were collected under a local surveillance program managed by veterinary authorities (Sanitary Veterinary and Food Safety Authority Timis County) and the Faculty of Veterinary Medicine (Timisoara, Romania). Sampled animals were treated according to the established standards for the humane care and use of animals specified in national legislation regarding animal welfare.[9]

In a cross-sectional study from January to December 2009, a total of 3651 blood samples were collected by jugular vein puncture of randomly selected animals from different groups: 1830 samples from sows and gilts from breeding farms, 226 samples from neonates, 179 samples from weaned pigs, and 1416 samples from grower-finisher pigs in 45 farms under the same ownership located in western Romania. In some cases clinical respiratory disease was observed. None of the animals were vaccinated against swine influenza. Tests conducted in this study represent a contractual obligation between our faculty and the owner of these farms. Within the herds, animals were individually identified and randomly selected for participation in the study. The number of samples was designed to guarantee a 95% probability of detecting at least one positive animal assuming a within-herd seroprevalence of 50%.

Samples were processed as they were submitted to the laboratory and tested using commercial enzyme-linked immunosorbent assays (ELISAs). For detection of antibodies against H1N1 and H3N2 subtypes, the Idexx SIV H1N1 Ab and Idexx SIV H3N2 Ab tests (Idexx Laboratories Inc, Westbrook, Maine), respectively, were used according

Infectious Diseases Department, Faculty of Veterinary Medicine Timisoara, Romania, Europe.

Corresponding author: Dr Corina Pascu, Faculty of Veterinary Medicine, Timisoara, Calea Aradului 119, 300645, Romania, Europe; Tel: 0040723277978; Fax: 0040256277187; E-mail: corina_pascu_ro@yahoo.co.uk.

This article is available online at aasv.org/shap.html.

Pascu C, Costinar L, Herman V. Enzyme-linked immunosorbent assay detection of antibodies against swine influenza virus in western Romania. *J Swine Health Prod.* 2012;20(2):87–90.

to the manufacturer's instructions.[10] Commercial ELISA kits are not available for the H1N2 subtype. For the H1N1 subtype, sample-to-positive (S:P) ratios < 0.40 are considered negative, and S:P ratios ≥ 0.40 are considered positive.[10] For the H3N2 subtype, S:P ratios < 0.30 are considered negative, S:P ratios ≥ 0.30 and < 0.40 are classified as suspect, and S:P ratios ≥ 0.40 are considered positive. A chi-square test was performed to observe a possible association between sow parity and seroprevalence rate.

In these tested herds, other respiratory pathogens contributed to respiratory disease. Most commonly, *Mycoplasma hyopneumoniae*, porcine reproductive and respiratory syndrome virus (PRRS), *Streptococcus suis,* and *Haemophilus parasuis* were detected. Samples for bacteriological culture (lung swabs) were collected from dead pigs. Organisms were identified at the Diagnostic Laboratory of the Faculty of Veterinary Medicine (Timisoara, Romania) and Pasteur Institute Department of Epidemiology and Diagnosis (Bucharest, Romania) using standard methodology.[11] Because of their fastidious nature, mycoplasmas were cultivated on special nutrient media supplemented with porcine serum (pleuropneumonia-like organism agar; Oxoid, Cambridge, United Kingdom). Indirect methods of detection were primarily used, ie, serological (Idexx *M. hyo.* Ab ELISA; Idexx Laboratories Inc) and molecular biology techniques (polymerase chain reaction; PCR). *Streptococcus* and *Haemophilus* species were cultivated on Columbia blood agar (Oxoid) with growth factor for *Haemophilus* (XV factor disks; Difco, Detroit, Michigan). PRRS was diagnosed by ELISA (HerdChek PRRS X3 Ab Test; Idexx Laboratories Inc) and detection of PRRS virus in tissues by PCR.

Results

Antibodies against H1N1 were identified among breeder and grower and finisher pigs, while antibodies against the H3N2 subtype were not identified in any tested samples (Table 1). Twenty of the 45 tested herds were seropositive. The highest proportion of serologically positive animals was recorded in sows (Table 1). Seroprevalence was highest in finisher pigs, followed by neonatal pigs, gilts, sows, grower pigs, and weaned pigs (Table 1). Seroprevalence was lowest in first and second parity sows (Table 2). There was a significant association between seroprevalence and parity, with greater seroprevalence in parities 5 and 6 than in parities 1 and 2 ($P < .01$). In the cold season (November through April), 220 of the 2065 samples tested (9.4%) were serologically positive for H1N1. In the warm season (May through October), 172 of the 1586 samples tested (9.2%) were positive for H1N1. Most serologically positive animals were identified in May and October (Figure 1).

Discussion

The standard method for detecting SIV antibodies is the hemagglutination inhibition (HI) test.[12] Recently, sensitive and specific SIV ELISA kits have been developed.[10,13,14] The ELISA method was chosen in this study because it is fast and less expensive to perform than the HI test, and we were interested in periodically monitoring the health status of swine herds.

The ELISA is considered the most sensitive serological assay for SIV, and ELISAs for differentiating antibodies to H1 and H3 have been developed.[15] At present, indirect ELISAs using H1N1 and H3N2 antigens are commercially available for serodiagnosis of SIV infection (Idexx Laboratories Inc).[15-17] Barbe et al,[13] in a study comparing the SIV ELISA and HI tests, determined that the ELISA had a specificity of 97% to 99% and a positive predictive value of 92% to 99%. Both tests are used in veterinary diagnostic laboratories and for seroprevalence studies worldwide.[13]

The SIV ELISA (especially the H3N2 ELISA), after further improvement, might be a valuable tool in detecting antibodies against influenza A viruses in general. Most other ELISAs are capable of detecting overlapping antigens (ie, antigenic overlap between vaccine and challenge strains), thus being powerful screening methods.[18]

Because of some weaknesses of the SIV H1N1 ELISA, researchers may prefer to use the hemagglutination inhibition test. The H1N1 ELISA uses an antigen prepared from classical H1N1 SIV, and therefore the range of swine H1 subtypes detected is limited.[14] In addition, the H1N1 test may miss recently infected animals, detecting only chronic infection.[14,15]

Other studies using ELISA tests have been conducted to determine seroprevalence of SIV subtypes. In the United States, researchers found an H1N1 subtype seroprevalence of 27.7%.[3] In Spain and Korea, seroprevalences were 30.6% and 39.12%, respectively.[19,20] In a study in pig farms in southern Romania between 2006 and 2009, blood samples collected from pigs aged 14 to 22 weeks were tested by ELISA (Idexx Laboratories Inc).[21] Results showed that 49% of animals were serologically positive for the H1N1 subtype and 67% for the H3N2 subtype.[21]

Swine influenza virus H1N1 is widespread in farms in western Romania. The small number of sera tested for H3N2 does not allow us to formulate a conclusion regarding the prevalence of this subtype. Further studies are needed, as it appears there are differences among Romanian swine herds. The differences between our results and those of Baraitareanu et al[21]are due to the different location of farms (southeast and west Romania). Our results for the H3N2 subtype were similar to those obtained in other countries, eg, the seroprevalence rate detected by ELISA for H3N2 was 0% in Poland and 0.1% in the Czech Republic,[2] and similar results were obtained in Lithuania.[18] On the other hand, in a 2001 study performed by ELISA in Bulgaria in grower and finisher pigs, no antibodies against H1N1 were detected, but seroprevalence of H3N2 was 96.7%.[22]

Some researchers[22,23] contend that there is a correlation between SIV antibody titer and season, but our study could not confirm this correlation. It has been reported that the highest incidence of SIV infections occurs during the winter months, between October and February.[24] Our results showed that most serologically positive animals were identified in May and October.

Implications

- Serological surveillance for SIV subtypes in pigs in western Romania is essential because this area has a large population of pigs, with the risk of new highly pathogenic subtypes.
- Cross-sectional studies are beneficial in supporting diagnostic findings in large commercial swine herds.

References

1. Gillespie T. Diagnosing endemic swine influenza virus in nursery pigs using cross-sectional serologic profiling. *Swine Health Prod*. 1999;7:81–83.

2. Van Reeth K, Brown IH, Dürrwald R, Foni E, Labarque G, Lenihan P, Maldonado J, Markowska-Daniel I, Pensaert M, Pospisil Z, Koch G. Seroprevalence of H1N1, H3N2 and H1N2 influenza Viruses in pigs in seven European countries in 2002–2003. *Influenza and Other Respiratory Viruses*. 2008;2:99–105. doi:10.1111/j.1750–2659.2008.00043.x.

Table 1: Presence of antibodies against swine influenza virus subtypes H1N1 and H3N2 in different age groups in 45 herds in western Romania*

Group	Age (weeks)	No. of sera	H1N1		H3N2	
			No. of positive herds (%)	No. of positive sera (%)	No. of positive herds (%)	No. of positive sera (%)
Neonatal	0-3	226	8 (17.7)	17 (7.5)	NT	NT
Nursery†	3-8	179	5 (11.1)	1 (0.6)	0 (0.0)	0 (0.0)
Grower	8-16	983	5 (11.1)	71 (7.2)	NT	NT
Finisher	17-26	433	10 (22.2)	18 (4.2)	0 (0.0)	0 (0.0)
Gilts	26-48	682	6 (13.3)	31 (4.6)	NT	NT
Sows	> 48	1148	5 (11.1)	254 (22.1)	0 (0.0)	0 (0.0)
Totals		3651	20 (44.4)	392 (10.8)	NA	NA

* Sera tested by enzyme-linked immunosorbent assay (Idexx SIV H1N1 Ab Test and Idexx SIV H3N2 Ab Test; Idexx Laboratories Inc, Westbrook, Maine). Samples with sample-to-positive ratio ≥ 0.40 considered positive. A herd was classified as seropositive if at least one animal was seropositive.

† Pigs were weaned at 21 days of age and moved out of the nursery when they were 8 weeks old.

NT = not tested; NA = not applicable.

3. Olsen CW, Carey S, Hinshaw L. Virologic and serologic surveillance for human, swine and avian influenza virus infections among pigs in the north-central United States. *Arch Virol*. 2000;145:1399–1419.

4. Olsen CW, Brown I, Easterday BC, Van Reeth K. Swine influenza. In: Straw BE, Zimmerman JJ, D'Allaire S, Taylor DJ. *Diseases of Swine*. 9th ed. Ames, Iowa: Iowa State University Press; 2006:469-482.

5. Brown IH, Harris PA, Alexander DJ. Serological studies of influenza viruses in pigs in Great Britain 1991–1992. *Epidemiol Infect*. 1995;114:511–520.

6. Olsen CW. Emergence of novel strains of swine influenza virus in North America. In: Morilla A, Yoon K-J, Zimmerman JJ, eds. *Trends in Emerging Viral Infections of Swine*. Ames, Iowa: Iowa State Press; 2002:37–44.

7. National Institute of Statistics. Press Releases Archive. Livestock existent on December 1, 2009. Available at: **http://www.insse.ro/cms/rw/pages/index.en.do**. Accessed 6 January 2012.

8. The National Sanitary Veterinary and Food Safety Agency [in Romanian]. Available at: **http://www.ansvsa.ro**. Accessed 13 December 2011.

9. Law no 8 from 2008, law amending and supplementing Law no 205 from 2004 on the animal protection. Chapter VI, Art. 7 and 22. Official Monitor no 29 from 2008. Lege pentru modificarea si completarea Legii nr. 205/2004 privind protectia animalelor [in Romanian]. Available at: **http://www.dreptonline.ro/monitorul_oficial/monitor_oficial.php?id_monitor=3034**. Accessed 13 December 2011.

10. Idexx Laboratories Inc. Swine Influenza Virus (SIV) Testing Solutions. Available at: **http://www.idexx.com/view/xhtml/en_us/livestock-poultry/swine/swine-influenza-virus.jsf**. Accessed 13 December 2011.

11. Quinn PJ, Markey BK, Carter ME, Donnelly WJ, Leonard FC. *Veterinary Microbiology and Microbial Disease*. Oxford, United Kingdom: Blackwell Science Ltd; 2002:49–54, 131–136.

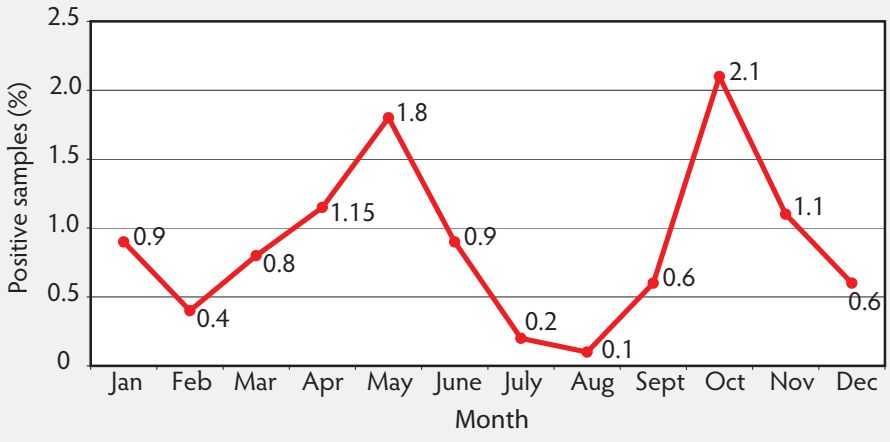
Table 2: Seroprevalence of antibodies* against H1N1 swine influenza virus detected in samples from sows of different parities in 45 western Romania herds

Parity	No of tested sera	No. of positive sera (%)†
1-2	383	61 (16.0)
3-4	390	90 (23.1)
5-6	375	103 (27.5)

* Sera tested by enzyme-linked immunosorbent assay (Idexx SIV H1N1 Ab Test; Idexx Laboratories Inc, Westbrook, Maine). Samples with sample-to-positive ratio ≥ 0.40 considered positive.

† Seroprevalence was higher in parities 5-6 than in parities 1-2 ($P < .01$; chi-square).

Figure 1: Seroprevalence of swine influenza subtype H1N1 antibodies by season. Blood samples were collected from pigs of different age groups, from neonatal piglets to sows, in 45 farms in western Romania**.** A total of 3651 samples were tested by enzyme-linked immunosorbent assay (Idexx SIV H1N1 Ab Test; Idexx Laboratories, Westbrook, Maine).



12. World Organization for Animal Health. Chapter 2.8.8: Swine Influenza. *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2011*. Available at: **http://www.oie.int/international-standard-setting/terrestrial-manual/access-online/**. Accessed 13 December 2011.

13. Barbe F, Labarque G, Pensaert M, Van Reeth K. Performance of a commercial Swine Influenza virus H1N1 and H3N2 antibody enzyme-linked immunosorbent assay in pigs experimentally infected with European influenza viruses. *J Vet Diag Invest*. 2009;21:88–96.

14. Yoon K-J, Janke BH, Swalla RW, Erickson G. Comparison of a commercial H1N1 enzyme-linked immunosorbent assay and hemagglutination inhibition test in detecting serum antibody against swine influenza viruses. *J Vet Diagn Invest*. 2004;16:197–201.

15. Yoon K-J, Janke BH. Swine influenza: Etiology, epidemiology, and diagnosis. In: Morilla A, Yoon K-J, Zimmerman JJ, eds. *Trends in Emerging Viral Infections of Swine*. Ames, Iowa: Iowa State Press; 2002:23–28.

*16. Perez CJ, Palacios AJ. Detection of SIV antibodies to classical H1N1, reassortant H1N1 and H3N2 in breeding and growing pigs in Jalisco Mexico State during 2006. *Proc IPVS.* Durban, South Africa. 2008;34.

17. Erickson GA, Gramer MR, Webby RJ. How to evaluate herds using swine influenza serology. *J Swine Health Prod*. 2005;13:222–224.

*18. Schmoll F, Lang C, Dürrwald R, Herwig V, Laue C, Sommerfeld-Stur I, Sipos W. Restrictions of commercial swine influenza ELISA kits for subtype-specific differentiation of swine influenza A-viruses. *Proc IPVS*. Copenhagen, Denmark. 2006;2:135.

19. Gutiérrez-Martín CB, Rodríguez-Delgado O, Álvarez-Nistal D, De la Puente-Redondo VA, García-Rioja F, Martín-Vicente J, Rodríguez Ferri EF. Simultaneous serological evidence of *Actinobacillus pleuropneumoniae*, PRRS, Aujeszky's disease and influenza viruses in Spanish finishing pigs. *Res Vet Sci*. 2000;69:9–13.

*20. Jeong K, Park Y-I, Jin W, Han J-H, Jeong H-K, Kim HJ, Rha J. Sero-prevalence of swine influenza virus in Korea. *Proc IPVS*. Hamburg, Germany. 2004;1:123.

21. Baraitareanu S, Bezman M, Marinache S, Cobzariu D, Bagrinovschi G, Otelea M. Campeanu MV, Ivana S, Danes D. Etiology and pathology in porcine respiratory disease complex (PRDC) in finishing pigs [in English]. The "Ion Ionescu de la Brad" University of Agricultural Sciences and Veterinary Medicine of Iasi, Romania. Scientific Works. 2010;53(12):920–926. Available at: **http://www.univagro-iasi.ro/simpozion_med/index.php/en/vol-53-4**. Accessed 6 January 2012.

22. Bochev I. Porcine respiratory disease complex (PRDC): A review. I. Etiology, epidemiology, clinical forms and pathoanatomical features. *Bulg J Vet Med*. 2007;10:131–146.

23. Loeffen WLA, Hunneman WA, Quak J, Verheijden JHM, Stegeman JA. Population dynamics of swine influenza virus in farrow-to-finish and specialised finishing herds in the Netherlands. *Vet Microbiol*. 2009;31:123–129.

24. Choi YK, Goyal SM, Joo HS. Retrospective analysis of etiologic agents associated with respiratory diseases in pigs. *Can Vet J*. 2003;44:735–737.

* Non-refereed references.