AVIAN DISEASES 52:498–501, 2008

Research Note—

Limited Susceptibility and Lack of Systemic Infection by an H3N2 Swine Influenza Virus in Intranasally Inoculated Chickens

Colleen Thomas,[A] Timofey B. Manin,[B] Artem V. Andriyasov,[B] and David E. Swayne[AC]

[A]Southeast Poultry Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, 934 College Station Road, Athens, GA 30605

[B]Federal Centre for Animal Health (FGI-ARRIAH), 600901 Yur'evets, Vladimir, Russia

Received 16 January 2008; Accepted and published ahead of print 19 April 2008

SUMMARY. Chickens were intranasally inoculated with the swine influenza virus (SIV) A/swine/NC/307408/04 (H3N2) (NC/04 SIV) to determine the infectivity of a North American SIV for chickens, as well as the possibility of chicken meat serving as a transmission vehicle for SIV. White leghorn (WL) layer-type chickens were used for initial pathotyping and infectivity tests, and a more comprehensive intranasal pathogenesis study was done with white Plymouth rock (WPR) broiler-type chickens. None of the NC/04 SIV-inoculated WL or WPR chickens displayed clinical signs. Serologic tests showed that the virus was able to infect both intranasally inoculated WL and WPR chickens, but the antibody titers were low, suggesting inefficient replication. Some of the NC/04 SIV-inoculated WL chickens shed low levels of virus, mostly from the alimentary tract, but viral shedding was not detected in NC/04 SIV-inoculated WPR chickens. The comprehensive pathogenesis study demonstrated that the virus did not cause systemic infections in WPR chickens, and feeding breast and thigh meat from the NC/04 SIV-inoculated WPR to WL chickens did not transmit NC/04 SIV.

RESUMEN. *Nota de Investigación*—Susceptibilidad limitada y carencia de infección sistémica en pollos inoculados intranasalmente con un virus H3N2 de influenza porcina.

Para determinar la infectividad en pollos de un virus Norteamericano de influenza porcina, así como la posibilidad de que la carne de pollo sirva como un vehículo de transmisión para el virus de influenza porcina, se inocularon pollos por vía intranasal con un virus H3N2 de influenza porcina designado A/cerdo/NC/307408/04 (por sus siglas en Inglés NC/04SIV). Para las pruebas iniciales de patotipificación e infectividad se utilizaron aves de la línea leghorn blanca y un estudio de patogenicidad más completo se realizó con aves de la línea de engorde (Plymouth rock). Ninguna de las aves leghorn blancas o Plymouth rock inoculadas con el virus NC/04SIV mostraron signos clínicos. Las pruebas serológicas demostraron que el virus fue capaz de infectar por vía intranasal a ambos tipos de aves, sin embargo, los títulos de anticuerpos fueron bajos sugiriendo una replicación ineficiente. Algunas de las aves leghorn blancas diseminaron bajos niveles del virus, predominantemente del tracto alimenticio. En las aves Plymouth rock inoculadas con el virus NC/04SIV no se detectó diseminación viral. El estudio completo de patogénesis demostró que el virus no causó infección sistémica en pollos Plymouth rock y que alimentar carne de pechuga y muslos proveniente de aves leghorn blancas y Plymouth rock inoculadas con el virus NC/04 SIV no transmitió el virus.

Key words: influenza, H3N2, swine, avian, chicken, meat

Abbreviations: AI = avian influenza; AIV = avian influenza virus(es); BHI = brain-heart infusion medium; DPI = days postinoculation; ECE = embryonating chicken egg; EID_{50} = 50% chicken embryo infective dose; GMT = geometric mean titer; H = hemagglutinin; HI = hemagglutinin inhibition; HPAIV = high pathogenicity avian influenza virus(es); HPNAI = high pathogenicity notifiable avian influenza; LPAI = low pathogenicity avian influenza; LPNAI = low pathogenicity notifiable avian influenza; N = neuraminidase; OIE = World Organization for Animal Health; SIV = swine influenza virus; SPF = specific-pathogen-free; WL = white leghorn; WPR = white Plymouth rock

Influenza A viruses cause natural infections in a variety of wild aquatic birds, poultry, pigs, horses, and humans (20). All influenza A viruses are categorized based on the major surface glycoproteins into 16 hemagglutinin (H1–16) and nine neuraminidase subtypes (N1–9). Influenza A viruses show host adaptation that restricts interspecies transmission, infectivity, and pathogenicity such that transmission and infectivity are more common between different bird species than between mammals and birds (18). However, H1 and H3 swine influenza viruses (SIV) are an exception, with frequent transfer from pigs to breeder or meat turkeys when both species are raised in geographic proximity. Such SIV infections have resulted in significant respiratory disease and egg production drops in turkeys (3,17,23). Reports of SIV infection in chickens have been infrequent and of unknown significance (16).

SIV causes acute respiratory disease in pigs. As with avian influenza virus (AIV) infections in birds, the severity of SIV infections in pigs depends on multiple factors, such as virus strain and host susceptibility (5). SIV is controlled primarily by vaccinating sows (24). Although vaccination greatly reduces viral replication and shedding, it does not necessarily prevent infection. Thus, SIV can circulate in a herd and infect pigs no longer protected by maternally derived antibodies. Until 1998, nearly all North American isolates were H1N1 with genetic and antigenic similarity to the "classical" H1N1 SIV first isolated in 1930 (13). Triple reassortant H3N2 SIV with gene segments of human, swine, and avian origin emerged in the U.S. swine population in 1998. Reassortment between new H3N2 SIV and classical H1N1 SIV led to the emergence of H1N2 SIV the following year. All three SIV subtypes continue to circulate

[C]Corresponding author. E-mail: david.swayne@ars.usda.gov

in the U.S. swine population, and new H3N1 isolates have recently been described (7,8).

AIV are categorized into low pathogenicity (LP) and high pathogenicity (HP) pathotypes based on virulence in chickens (20). LPAIV replication is typically restricted to the respiratory and/or digestive tract, and infections are usually mild in the absence of co-infecting pathogens or other exacerbating conditions. In contrast, HPAIV cause severe systemic disease with replication in multiple visceral organs and production of high mortality. Infected poultry products such as meat can serve as transmission vehicles for HPAIV to poultry. For example, epidemiologists have concluded that imported fresh turkey meat was the most likely source of the H5N1 HPAIV that caused the January 2007 outbreak on a turkey farm in Suffolk, Great Britain (4). Because HPAIV are present in multiple tissues, including skeletal muscle (10,19), the risk of spreading AIV through trade of poultry products is higher for HPAIV than for LPAIV (12,26). Therefore, knowledge of the pathogenesis of influenza virus infections in commercial poultry is a critical component of science-based risk assessment.

For trade and control purposes, certain avian influenza (AI) infections in poultry are notifiable to the World Organization for Animal Health (OIE). High pathogenicity notifiable influenza (HPNAI) includes all HPAIV, whereas low pathogenicity notifiable avian influenza (LPNAI) includes all H5 and H7 LPAI viruses. LPAI of the H1–4, H6, and H8–16 subtypes and swine influenza are not notifiable to the OIE. The OIE, which is responsible for international sanitary standards under the World Trade Organization, recommends that poultry products from HPNAI-affected countries, zones, or compartments be processed to ensure destruction of AI virus before export (12). Other than isolation of low titers of H9N2 LPAIV from chicken meat and bone marrow imported into Japan from China (6,9), isolation of LPAIV from poultry products has not been reported in the literature. However, LPAIV from respiratory secretions or feces could be a source of carcass contamination. In a previous study, one H7 LPNAI virus strain was isolated from body cavity rinses of infected chickens both before and after the carcasses were rinsed in water with 30 ppm chlorine and chilled (19). However, virus was not detected in breast and thigh meat from these same carcasses, suggesting that the virus was present in the residual lung and air sac tissues in the body cavity.

The current study was done to assess the infectivity of a North American SIV (A/swine/NC/307408/04 [H3N2]) for chickens and to determine the relative risk of spreading SIV through trade in chicken meat.

MATERIALS AND METHODS

Virus strain and virologic methods. The SIV strain A/swine/NC/307408/04 (H3N2) (NC/04 SIV) was passaged in 10-to-11-day-old embryonating chicken eggs (ECEs). Amnioallantoic fluid was harvested 3 days after allantoic sac inoculation and used at 1:10 dilution (intravenous pathogenicity test) or diluted to 10^6 mean 50% chicken embryo infective dose (EID_{50}) in brain-heart infusion medium (BHI) for infectivity and pathogenesis studies.

Oropharyngeal and cloacal swabs were collected and placed in 1.5 ml of BHI containing the appropriate antibiotics (22). Swabs and tissue samples were stored at −70 C until virus isolation and titration were performed in ECEs according to standard protocols (22). Blood was collected and separated into fractions as described previously (19). For all samples, virus in ECE allantoic fluid was detected by the hemagglutination assay, and the 50% endpoints for titrations were calculated by the Reed and Muench method (25).

Chickens. Specific-pathogen-free (SPF) white leghorn (WL) layer-type and white Plymouth rock (WPR) broiler-type chicken strains from our in house flocks were maintained in negative pressure high-efficiency particulate air-filtered stainless steel isolation cabinets under constant illumination within a biosafety level 2 enhanced facility. Food and water were provided *ad libitum*.

Experimental design. *Experiment 1. Pathogenicity and infectivity of NC/04 SIV virus for chickens*. Eight 4-week-old WL chickens were each inoculated intravenously with a 1:10 dilution of allantoic fluid containing NC/04 SIV (back titer, $10^{5.8}$ EID_{50}) in 0.2 ml of BHI as prescribed for regulatory pathogenicity testing (22). To determine infectivity by a simulated natural route of exposure, eight 4-week-old WL chickens were each inoculated intranasally with 10^6 EID_{50} of NC/04 SIV (back titer, $10^{5.9}$ EID_{50}) in 0.1 ml of BHI. Oropharyngeal and cloacal swab samples were taken from five of these chickens on 1, 3, and 5 days postinoculation (DPI). For both groups, morbidity and mortality were recorded daily until the birds were euthanatized on 10 DPI with intravenous pentobarbital (100 mg/kg body weight). Serum was collected from each chicken for serologic testing preinoculation and before euthanasia.

Experiment 2 Pathogenesis of intranasal NC/04 SIV virus infection in broilers. Four-week-old WPR chickens were each inoculated with 10^6 EID_{50} NC/04 SIV (back titer, $10^{6.1}$ EID_{50}) in 0.1 ml of BHI ($n = 30$) or an equivalent volume of uninfected allantoic fluid ($n = 10$). Five virus-inoculated WPR chickens were sampled on 1, 2, 3, 4, 7, and 10 DPI, and five sham-inoculated WPR chickens were sampled on 3 and 10 DPI. The following samples were taken from each WPR chicken for virus isolation: blood (plasma, white blood cells, and red blood cells), swabs (air sac, oropharyngeal, and cloacal), lung, trachea, skeletal muscle (breast and thigh), and bone (from the pelvic girdle). In addition, an average of 11.1 g of breast meat and 5.7 g of thigh meat for use in Experiment 3 were harvested from each of the virus-inoculated and sham-inoculated WPR chickens euthanized on 3 DPI, and the body cavity of each of these WPR chickens was rinsed with 3 ml of BHI, which was saved for virus isolation. Serum from each of the 40 WPR chickens was collected for serologic testing preinoculation and from each of 10 WPR chickens before euthanasia on 10 DPI (intravenous pentobarbital, 100 mg/kg).

Experiment 3. Transmission by feeding meat from NC/04 SIV-inoculated WPR chickens. From Experiment 2, meat harvested from the intranasal-NC/04 SIV or sham-inoculated WPR chickens on 3 DPI was finely chopped. After fasting for 12 hours, groups of 10 4-week-old WL chickens were fed pooled breast and thigh meat from sham-inoculated chickens (83.2 g total), pooled breast meat from NC/04 SIV-inoculated chickens (57.2 g total), or pooled thigh meat from NC/04 SIV-inoculated chickens (27.4 g total). When the chickens were next observed (5 hr after feeding time), all of the meat had been consumed. Serum was taken from each chicken on 14 days postfeeding for serologic testing.

Serology. For all experiments, serum was tested for influenza A antibodies by both the hemagglutinin inhibition (HI) assay using NC/04 SIV as antigen and the agar-gel precipitin test (22). All of the preinoculation serologic tests were negative for influenza A and H3 HI antibodies.

RESULTS

In the current study, none of the WL chickens inoculated with NC/04 SIV intravenously or intranasally developed clinical signs or died (Table 1), indicating the virus was LP for chickens. Serologic tests for H3 antibodies determined that all of the intranasally inoculated WL chickens had been infected with NC/04 SIV. However, the HI titers were very low, suggesting the virus replicated inefficiently in WL chickens. This was substantiated by low peak virus replication titers from oral and cloacal swabs ($10^{1.0}$ and $10^{1.2}$ EID_{50}, respectively) compared with oral and cloacal swabs from WPR chickens intranasally infected with two chicken-adapted H7N2 LPAI viruses ($10^{5.5}$ and $10^{4.3}$ EID_{50}, respectively) (19). Furthermore, the pathogenesis of the infection by the SIV versus LPAIV differed, with the SIV infection being primarily alimentary,

Table 1. Pathotyping of A/swine/NC/307408/04 (H3N2) in 4-week-old WL chickens.

			Virus isolation from swabs (log_{10} EID_{50}/ml)[A]							
			1 DPI		3 DPI		5 DPI		Serology	
Infection route	Morbidity	Mortality	Oral	Cloacal	Oral	Cloacal	Oral	Cloacal	AGP[B]	HI (GMT)
Intravenous	0/8	0/8	NA[C]	NA	NA	NA	NA	NA	NA	NA
Intranasal	0/8	0/8	0/5	2/5 (1.0)	1/5 (1.0)	3/5 (1.2)	0/5	3/5 (1.1)	0/8	8/8 (12)

[A]Virus isolation performed on five intranasally infected chickens. The average titer of positive samples is shown in parentheses. The detection limit of the assay is 0.9 log_{10} EID_{50}/ml.
[B]NA = not applicable.
[C]AGP = agar gel precipitation.

whereas the LPAIV infections were primarily respiratory. The low levels of virus isolated from swab samples and the low HI titers obtained for NC/04-SIV-infected chickens indicate that NC/04 SIV did not replicate efficiently in the WL chickens.

Likewise, none of the sham-inoculated or NC/04 SIV-inoculated WPR (broiler-type) chickens displayed clinical signs after intranasal inoculation. At 10 DPI, 60% of the NC/04 SIV-inoculated WPR chickens had H3 HI antibodies, but with low titers (geometric mean titer [GMT] = 16) comparable with those seen in the WL chickens in Experiment 1. However, virus was not isolated from any swab samples, blood, tissue, breast or thigh meat, or body cavity rinses of the sham or NC/04 SIV-inoculated WPR chickens. As expected given the inability to isolate virus from these meat samples, none of the WL chickens that were fed meat from NC/04 SIV-inoculated or sham-inoculated WPR chickens displayed clinical signs during the 14-day observation period, nor seroconverted at the end of the study.

DISCUSSION

The emergence of triple reassortant SIV in North America in the late 1990s (which contained genome segments from swine, avian, and human influenza strains) (13) raises the question of whether North American SIV strains can infect avian species, and whether commercial poultry products could serve as transmission vehicles for these viruses. Transmission of SIV to turkeys is not uncommon, and infections of turkeys in the United States with H3N2 (3,23) or H1N2 (17) reassortant swine influenza viruses have been reported. A swine-like reassortant H1N2 strain was isolated from a wild duck in the United States (14), indicating that other avian species may be susceptible to these viruses.

However, natural infections with swine-like influenza viruses seem to be uncommon in chickens (16). Campitelli *et al.* (1) showed that H3N2 isolates from pigs in Italy failed to replicate in experimentally infected 3-week-old SPF WL chickens inoculated intravenously or intranasally/intratracheally/orally. In contrast, the current study shows that a recent North American H3N2 SIV isolate can replicate in chickens, albeit inefficiently. The genetic and antigenic properties of European SIV strains differ from those of North American SIV strains of the same subtype (24), and this could explain differences in infectivity for chickens.

Susceptibility of chickens to infection with AIV depends on several factors, including chicken breed and age. A previous study with an H4N8 LPAIV isolated from layer chickens produced more severe lesions in commercial and SPF WL chickens than in 5-week-old commercial broiler chickens (21), suggesting that WL chickens are more susceptible than broiler chickens to this LPAIV strain. In the current study, SIV infections were subclinical in intranasally inoculated WPR and WL chickens, and low HI titers indicated inefficient virus replication and infection in both breeds. However, the isolation of NC/04 SIV from WL swab samples but not WPR swab samples suggests that WL chickens may be more susceptible to SIV.

AIV has been isolated from or demonstrated in meat obtained from chickens naturally or experimentally (intranasally) infected with various HPAIV (2,10,11,15,19), but LPAIV are not usually found in chicken meat (10,19). In a previous experimental study, WL chickens fed meat from H5N1 HPAIV-infected WPR chickens became infected and died, whereas WL chickens fed meat from H5N2 HPAIV- or H7N2 LPAIV-infected WPR chickens did not become infected (19). The meat from the H7N2 LPAIV-infected chickens lacked AIV based on failed attempts to isolate virus in ECEs. AIV was present in the meat from H5N2 HPAIV-infected chickens, but at levels too low to produce infection by consumption. The failure to isolate SIV from the meat and other organs of chickens infected with NC/04 SIV during the current study indicates that SIV infections are not systemic in chickens and that the risk of spreading this virus through trade of poultry products is even lower than that for LPAIV.

In summary, chickens could be infected with a North American SIV after simulated natural respiratory exposure, but the virus replicated inefficiently in this host, primarily in the digestive tract. The pathogenesis of NC/04 SIV in chickens was similar to that for LPAIV, but the levels of SIV shedding were much lower and SIV was less consistent at respiratory replication compared with LPAIV. Together, the data suggest that the risk of spreading SIV through trade of chicken meat or related products is extremely low.

REFERENCES

1. Campitelli, L., C. Fabiani, S. Puzelli, A. Fioretti, E. Foni, A. De Marco, S. Krauss, R. G. Webster, and I. Donatelli. H3N2 influenza viruses from domestic chickens in Italy: an increasing role for chickens in the ecology of influenza? J. Gen. Virol. 83:413–420. 2002.

2. Capua, I., C. Terregino, G. Cattoli, F. Mutinelli, and J. F. Rodriguez. Development of a DIVA (differentiating infected from vaccinated animals) strategy using a vaccine containing a heterologous neuraminidase for the control of avian influenza. Avian Pathol. 32:47–55. 2003.

3. Choi, Y. K., J. H. Lee, G. Erickson, S. M. Goyal, H. S. Joo, R. G. Webster, and R. J. Webby. H3N2 influenza virus transmission from swine to turkeys, United States. Emerg. Infect. Dis. 10:2156–2160. 2004.

4. Department for Environmental, Food, and Rural Affairs, National Emergency Epidemiology Group, Food and Farming Group, London, England. Outbreak of highly pathogenic H5N1 avian influenza in Suffolk in January 2007. Department for Environmental, Food, and Rural Affairs, London, England. pp. 1–32. 2007. [cited 2007 April 4]. Available from: www.defra.gov.uk/animalh/diseases/notifiable/disease/ai/pdf/epid_findings 050407.pdf.

5. Easterday, B. C., and K. Van Reeth. Swine influenza. In: Diseases of swine. B. E. Straw, S. D'Allaire, W. L. Mengeling, and D. J. Taylor, eds. Iowa State University Press, Ames, IA. pp. 277–290. 1999.

6. Kishida, N., Y. Sakoda, M. Eto, Y. Sunaga, and H. Kida. Co-infection of *Staphylococcus aureus* or *Haemophilus paragallinarum* exacerbates

H9N2 influenza A virus infection in chickens. Arch. Virol. 149:2095–2104. 2004.

7. Lekcharoensuk, P., K. M. Lager, R. Vemulapalli, M. Woodruff, A. L. Vincent, and J. A. Richt. Novel swine influenza virus subtype H3N1, United States. Emerg. Infect. Dis. 12:787–794. 2006.

8. Ma, W., M. Gramer, K. Rossow, and K. J. Yoon. Isolation and genetic characterization of new reassortant H3N1 swine influenza virus from pigs in the midwestern United States. J. Virol. 80:5092–5096. 2006.

9. Mase, M., M. Eto, K. Imai, K. Tsukamoto, and S. Yamaguchi. Characterization of H9N2 influenza A viruses isolated from chicken products imported into Japan from China. Epidemiol. Infect. 135:386–391. 2007.

10. Mo, I. P., M. Brugh, O. J. Fletcher, G. N. Rowland, and D. E. Swayne. Comparative pathology of chickens experimentally inoculated with avian influenza viruses of low and high pathogenicity. Avian Dis. 41:125–136. 1997.

11. Moses, H. E., C. A. Brandly, E. E. Jones, and E. L. Jungherr. The isolation and identification of fowl plague virus. Am. J. Vet. Res. 9:314–328. 1948.

12. Office International des Epizooties. 2007. Terrestrial Animal Health Code. [cited 2007 April 4]. Available from: http://www.oie.int/eng/normes/mcode/en_sommaire.htm

13. Olsen, C. W. The emergence of novel swine influenza viruses in North America. Virus Res. 85:199–210. 2002.

14. Olsen, C. W., A. Karasin, and G. Erickson. Characterization of a swine-like reassortant H1N2 influenza virus isolated from a wild duck in the United States. Virus Res. 93:115–121. 2003.

15. Purchase, H. S. Experiments on the viability of the virus of fowl-plague under trade conditions. Vet. Rec. 11:644–648. 1931.

16. Senne, D. A. National Veterinary Services Laboratory activities. In: Proc. 110th annual meeting of the United States Animal Health Association, Minneapolis, MN. pp. 634–637. 2006.

17. Suarez, D. L., P. R. Woolcock, A. J. Bermudez, and D. A. Senne. Isolation from turkey breeder hens of a reassortant H1N2 influenza virus with swine, human, and avian lineage genes. Avian Dis. 46:111–121. 2002.

18. Swayne, D. E. Epidemiology of avian influenza in agricultural and other man-made systems. In: Avian influenza. D. E. Swayne, ed. Blackwell Publishing, Ames, IA. pp. 59–85. 2008.

19. Swayne, D. E., and J. R. Beck. Experimental study to determine if low-pathogenicity and high-pathogenicity avian influenza viruses can be present in chicken breast and thigh meat following intranasal virus inoculation. Avian Dis. 49:81–85. 2005.

20. Swayne, D. E., and D. A. Halvorson. Influenza. In: Diseases of poultry, 12th ed. Y. M. Saif, J. R. Glisson, A. M. Fadly, L. R. McDougald, and L. Nolan, eds. Blackwell Publishing, Ames, IA. In press. 2008.

21. Swayne, D. E., M. J. Radin, T. M. Hoepf, and R. D. Slemons. Acute renal failure as the cause of death in chickens following intravenous inoculation with avian influenza virus A/chicken/Alabama/7395/75 (H4N8). Avian Dis. 38:151–157. 1994.

22. Swayne, D. E., D. A. Senne, and C. W. Beard. Avian influenza. In: Isolation and identification of avian pathogens. D. E. Swayne, J. R. Glisson, M. W. Jackwood, J. E. Pearson, and W. M. Reed, eds. American Association of Avian Pathologists, Kennett Square, PA. pp. 150–155. 1998.

23. Tang, Y., C. W. Lee, Y. Zhang, D. A. Senne, R. Dearth, B. Byrum, D. R. Perez, D. L. Suarez, and Y. M. Saif. Isolation and characterization of H3N2 influenza A virus from turkeys. Avian Dis. 49:207–213. 2005.

24. Thacker, E., and B. Janke. Swine influenza virus: zoonotic potential and vaccination strategies for the control of avian and swine influenzas. J. Infect. Dis. 197(Suppl. 1):S19–S24. 2008.

25. Villegas, P. Titration of biological suspensions. In: Isolation and identification of avian pathogens. D. E. Swayne, J. R. Glisson, M. W. Jackwood, J. E. Pearson, and W. M. Reed, eds. American Association of Avian Pathologists, Kennett Square, PA. pp. 248–254. 1998.

26. Zepeda, C., and M. D. Salman. Assessing the probability of the presence of low pathogenicity avian influenza virus in exported chicken meat. Avian Dis. 51:344–351. 2007.

ACKNOWLEDGMENTS

We thank J. Beck, J. Doster, and K. Moresco for excellent technical assistance. This research was supported by CRIS projects 6612-32000-039-00D and 6612-32000-048-00D.