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EXPOSURE TO SWINE H1 AND H3 AND AVIAN H5 AND H9 INFLUENZA A VIRUSES AMONG FERAL SWINE IN SOUTHERN CHINA, 2009

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ABSTRACT: Swine play an important role in the disease ecology of influenza. Swine may provide the potential for mixed infections and genetic reassortment between avian, human, and porcine influenza viruses. We investigated the prevalence of antibodies to swine H1 and H3 influenza viruses and avian H5 and H9 influenza viruses in feral swine in southern China. Serum samples were collected from 31 feral swine harvested in 2009 in southern China. Of 31 serum samples tested, 14 (45%) had detectable antibody to H1 influenza virus and 23 (74%) were positive for H3 subtype. The antibody prevalence against both the swine H1 virus and the swine H3 virus was 45% (14/31). Five samples were reactive with both H1 and N1 subtype viruses, suggesting exposure to H1N1 viruses. All the sera tested were negative for avian H5 and H9 influenza viruses. Further investigations of influenza virus exposure of feral swine are needed to clarify their role in influenza ecology.

Key words: Avian influenza, feral swine, influenza, seroprevalence, southern China, *Sus scrofa*, swine influenza.

INTRODUCTION

Influenza A virus infects numerous species of birds and a diversity of mammals (including cetaceans and humans), causing enormous economic loss and public health problems. Swine (*Sus scrofa*) possess both α(2, 3)- and α(2, 6)-linked sialic acid, which are well recognized by avian and human influenza viruses, respectively (Ito et al., 1998). Swine can be infected with multiple influenza subtypes and may serve as "mixing vessels" for mixed infections and potential genetic reassortment among avian, human, and porcine influenza viruses (Kida et al., 1994; Ito et al., 1998). Currently, H1N1, H1N2, and H3N2 influenza viruses are the main subtypes cocirculating in swine populations worldwide (Yu et al., 2007; Chutinimitkul et al., 2008; Van Reeth et al., 2008). H5 and H9 avian influenza infections were also detected in swine in China (Ninomiya et al., 2002). Swine may play an important role in the disease ecology of influenza, and may be a source of new pandemic virus strains, especially in regions where avian influenza virus is circulating.

Despite increasing knowledge of influenza virus dynamics in wild birds, the viral circulation in feral swine remains largely unknown (Vittecoq et al., 2012). Only a few investigators have examined exposure of feral swine to influenza viruses (Kaden et al., 2008; Closa-Sebastia et al., 2011; Vittecoq et al., 2012). Direct detection of viral antigen in feral swine is difficult because of the short course of the infection. Therefore serologic investigations generally dominate epidemiologic surveillance in feral swine. We report the prevalence of antibody to swine H1 and H3 influenza viruses in feral swine in southern China and investigated whether avian H5 and H9 influenza transmission occurred in feral swine from southern China by detecting serum antibodies with the use of hemagglutination inhibition assay. Our results will help evaluate the risks that feral swine may pose to the swine industry and to human health.

MATERIALS AND METHODS

Sample collection

Study sites were located in Zengcheng county, within Guangzhou city, Guangdong Province, southern China (23°7′48″N, 113°48′36″E). Blood was collected from 31 feral swine that were harvested between August and November 2009. Blood was allowed to clot, and centrifuged to separate serum, which was stored at −20 C until analyses. With the use of tooth eruption patterns, animals were grouped into two age classes: yearlings and subadaults (<24 mo old), and adults (≥24). Among the 31 serum samples, three were collected from yearlings and subadaults and 28 were from adults, including 27 females and 1 male. Animal handling and trapping were performed following Institutional Animal Care and Use Committee protocols, guidelines, and approval, and were collected as part of a legal harvest.

Viruses and cells

Four influenza viruses (NYMCX-179A conventional reassortant virus [H1N1], A/Swine/Guangdong/7/2005 [H3N2], A/environment/Qinghai/1/2008 [H5N1], and A/black-billed magpie/Guangxi/29/2005 [H9N2]) from the repository of our laboratory were propagated in the allantoic cavities of 9-day-old embryonated chicken eggs at 37 C for 3 days. Madin-Darby canine kidney (MDCK) cells were grown in Dulbecco's modified eagle medium (DMEM) supplemented with 10% bovine calf serum (Gibco, Life Technologies, New York, New York, USA) and used for the neutralization test (NT).

Sample testing

Each serum sample was tested against chicken red blood cells (RBCs) in the absence of virus to rule out nonspecific hemagglutination and treated by adsorption of the serum with chicken RBCs to inhibit the agglutination. This was done by adding 0.025 ml of packed chicken RBCs to each 0.5 mL of antiserum, mixing gently, and leaving for 30 min; RBCs were pelleted by centrifugation at 800 × G for 5 min, and the adsorbed sera were decanted (World Organisation for Animal Health [OIE], 2011).

Antibodies against influenza virus H1, H3, H5, and H9 subtypes were detected by hemagglutination inhibition (HI) according to OIE recommendations (OIE, 2011). Briefly, 25 μL of serial twofold dilutions of the treated serum samples were mixed with 4 hemagglutinating units (HAU) of virus in microtiter plates and incubated at room temperature for 30 min. Fifty microliters of 0.5% chicken RBCs was added to each well and incubated at room temperature for 30 min. The HI titer was the highest dilution of serum causing complete inhibition of 4 HAU of antigen. HI titers were regarded as positive if there was inhibition at a serum dilution of 1/16 (2^4 or $\log_2 4$ when expressed as the reciprocal) or more against 4 HAU of antigen.

Neutralization tests to detect hemagglutinin (HA) subtype-specific antibody are often carried out in parallel with HI to supplement the reliability of the HI assay, especially for detecting antibodies to avian influenza viruses in mammalian serum (Jung et al., 2007). We used NT to confirm that sera negative to H5 or H9 by the HI were truly negative. The NT using cell culture was performed according to Kida et al. (1982). Serial dilutions of receptor destroy enzyme (RDE) –treated sera were mixed with 10^2 median tissue-culture infective dose ($TCID_{50}$) of virus and incubated at 37 C for 1 hr. The virus and serum mixture was inoculated onto confluent MDCK cell monolayers in 96-well plates, and incubated at 37 C in 5% CO_2. After 1 hr, the inoculum was removed and 100 μL of DMEM was added to each well. Cells were incubated at 35 C in 5% CO_2 for 2 days. The neutralization titer was determined as the reciprocal of the serum dilution that caused 50% inhibition of cytopathic effect.

We used the conventional World Health Organization (WHO) neuraminidase inhibition (NAI) test (WHO, 2011). Serum samples were inactivated at 56 C for 30 min and diluted 1:10 before use. The NAI titer of an antiserum is defined as the dilution giving 50% inhibition of NA activity (NAI_{50}).

Viral isolation followed Zhou et al. (2008). Sera (200 μL) were inoculated into the allantoic sac of five embryonating, 9-day-old, specific-pathogen-free chicken eggs, and the eggs were incubated at 37 C for 3 days. Eggs containing dead or dying embryos (as they arose) and all eggs remaining at the end of the incubation period, were chilled to 4 C, and the allantoic fluids were tested with hemagglutination and HI.

RESULTS

Upon examination we found a very low cross-reactivity between the reference antigen and antibodies used in the study. Of the 31 serum samples, 14 (45%) were positive for H1 influenza virus, ranging from serum dilution of $1{:}2^4$ to $1{:}2^{10}$ (Table 1). Twenty-

TABLE 1. Serologic test results for antibodies against subtypes of influenza virus in feral swine (*Sus scrofa*) in Guangdong, China.

Sample no.	Hemagglutination inhibition titers for four subtypes of influenza viruses				Neuraminidase (NA) subtypes	
	H1	H3	H5	H9	N1	N2
1	Negative	Negative	Negative	Negative	Negative	Negative
2	Negative	Negative	Negative	Negative	Negative	Negative
3	Negative	Negative	Negative	Negative	Negative	Negative
4	Negative	Negative	Negative	Negative	Negative	Negative
5	Negative	Negative	Negative	Negative	Negative	Negative
6	Negative	64	Negative	Negative	Negative	Positive
7	Negative	128	Negative	Negative	Negative	Positive
8	Negative	128	Negative	Negative	Negative	Positive
9	Negative	Negative	Negative	Negative	Negative	Negative
10	Negative	128	Negative	Negative	Negative	Positive
11	1,024	64	Negative	Negative	Positive	Positive
12	64	64	Negative	Negative	Negative	Positive
13	16	128	Negative	Negative	Negative	Positive
14	128	64	Negative	Negative	Negative	Positive
15	64	64	Negative	Negative	Negative	Positive
16	32	256	Negative	Negative	Negative	Positive
17	128	128	Negative	Negative	Positive	Positive
18	128	64	Negative	Negative	Negative	Positive
19	64	512	Negative	Negative	Negative	Positive
20	64	64	Negative	Negative	Positive	Positive
21	Negative	Negative	Negative	Negative	Negative	Negative
22	Negative	Negative	Negative	Negative	Negative	Negative
23	Negative	32	Negative	Negative	Negative	Positive
24	64	64	Negative	Negative	Positive	Positive
25	64	64	Negative	Negative	Positive	Positive
26	Negative	32	Negative	Negative	Negative	Positive
27	32	128	Negative	Negative	Negative	Positive
28	Negative	128	Negative	Negative	Negative	Positive
29	Negative	128	Negative	Negative	Negative	Positive
30	32	256	Negative	Negative	Negative	Positive
31	Negative	128	Negative	Negative	Negative	Positive
Positive no. (%)	14 (45.2)	23 (74.2)	0	0	5 (16.13)	23 (74.2)

three samples (74%) were positive for H3 influenza virus, ranging from a serum dilution of 1:2^5 to 1:2^9. Among the 23 H3-antibody–positive samples, 14 (45%) were positive to both H1 and H3 subtype influenza, and 8 (26%) were only positive to H3 influenza. The proportion of animals with antibodies against the H3 subtypes (74%) was higher than the proportion with antibodies to H1 subtypes (45%).

The NAI tests were performed to differentiate between exposures to H1N1 and H1N2. Among the 14 H1-antibody–positive samples, 5 were positive to N1 subtype by NAI, suggesting exposure to H1N1. These 14 samples were all N2 positive by the NAI test. However, it is difficult to discern whether the N2 antibodies came from H1N2 or H3N2 viruses with the use of this method.

Antibodies against influenza virus of H5 and H9 subtypes were not detected by HI or NT tests (Table 1). No influenza virus was isolated from the samples, likely because viremia is usually absent or very low when samples are collected.

DISCUSSION

Influenza A virus expresses two major surface glycoproteins, the hemagglutinin (HA) and neuraminidase (NA). The HA

glycoprotein plays an important role in the initial stages of infection: receptor binding and the fusion of virus and cell membranes (Skehel and Wiley, 2000). The HA subtypes are classified into two groups based on their antigenic properties and their major structural features (Medina and Garcia-Sastre, 2011). Group 1 encompasses the H1a (which include the H5 HA subtype), H1b and H9 clades, and group 2 consists of the H3 and H7 clades (Medina and Garcia-Sastre, 2011); HA is the major antigenic driver of the adaptive immune response (Johansson et al., 1987; Johansson and Kilbourne, 1994). The dominant immune response against influenza HA is thought to be directed to the head of the glycoprotein, specifically to defined antigenic regions that surround the receptor binding pocket. Antibodies against the conserved stalk region of the HA, though less abundant, can be cross-reactive between HA subtypes within the same phylogenetic group (Pica et al., 2012). In this study, all of the H1-antibody–positive individuals were positive for H3 and all the samples were negative for H5 and H9. According to the phylogenetic analysis of HA, if the results were from cross-reactivity, it is more likely that those samples were also H5- and H9-antibody positive, which was discordant with the test results. Therefore, it is not likely that the cross-reactivity was caused by antistalk antibodies and the antibody specific for H1 and H3 were present in the serum samples.

Previous studies suggested that little cross-reactivity between H1 and H3 influenza viruses occurred during antibody prevalence investigations in swine (Jung et al., 2007). In a comparison of three serologic assays carried out to determine the cross-reactivity of antibodies from eight genetically diverse US swine influenza viruses, the sample-to-positive-control ratio was low when the H1 kit was used to detect H3 exposure serum (Leuwerke et al., 2008). These studies suggest that our results accurately reflect the exposure of feral swine to different subtypes of influenza viruses.

However, HI may not detect all the neutralization antibodies during influenza infection (Kida et al., 1982; Lu et al., 1982). Antibodies that failed to inhibit hemagglutination may bind nearer to the hydrophobic end of the HA molecule and might not block the receptor binding site for erythrocytes, but may still inhibit infectivity by interference with cell fusion or viral replication (Kida et al., 1982). Thus, the NT was carried out in parallel with the HI test to detect HA subtype-specific antibodies to influenza viruses in sera. In our samples, the NT test results were accordant with HI test results.

Feral swine are resident in many countries, and pose ecologic and infectious disease concerns. They harbor many important infectious agents that are transmissible to domestic pigs and other animal species, including humans (Meng et al., 2009). Migration of human populations to suburban areas due to increasing population density, increased use of land for agriculture, and deforestation have all increased chances of contact of humans and domestic animals with wild boars.

Feral swine are considered a major reservoir of H1N1, H1N2, and H3N2, and can be experimentally infected by highly pathogenic H5N1 virus (Ruiz-Fons et al., 2008). Influenza infections in wild boars were reported in the United States (Gipson et al., 1999; Corn et al., 2009), France (Vittecoq et al., 2012), Germany (Polley et al., 2007; Kaden et al., 2008, 2009), Poland (Markowska-Daniel and Pejsak, 1999; Markowska-Daniel and Kowalczyk, 2005), and Spain (Vicente et al., 2002; Closa-Sebastia et al., 2011). Our survey is consistent with previous investigations showing that coinfection of H1 and H3 influenza viruses is common in feral swine. H1N1 is the most prevalent subtype detected in wild boar populations in Europe (Vittecoq et al., 2012), whereas H3 seems to be the most prevalent

subtype in feral swine in southern China. Most investigations in European countries and the United States reported lower antibody prevalence in wild boar than our study (Markowska-Daniel and Pejsak, 1999; Vicente et al., 2002). Investigations of influenza exposure in feral swine should be more widely conducted to determine if the pattern we observed is normal.

Influenza can be transmitted reciprocally between humans, domestic animals (such as swine and poultry), and wildlife, where they come into contact. Swine influenza reassortant H1N2 has been isolated from a wild duck (Olsen et al., 2003). Coinfection by several strains in swine may provide the potential for reassortment with the generation of new combinations of genetic features, new subtypes, and even the potential for new pandemic variants. The H3N2 subtype widespread in US swine, which has genes from human lineages (HA, NA, PB1), swine lineages (NS, M, NP), and avian lineages (PB2, PA), was generated through reassortment (Webby et al., 2000). The potential for feral swine to become infected with avian, swine, or human influenza, to act as recombination vessels, and to transport and transmit virus to agricultural and human sites is significant.

Our findings suggest that swine influenza subtypes H1 and H3 are circulating in feral swine populations with a high prevalence. However, it seems that the subtype H3 presently is more predominant in feral swine than the subtype H1. Although no samples were detected with antibody to avian influenza H5 and H9 subtypes, we cannot exclude that low or occasional avian influenza circulation took place during the study. Influenza exposure investigations in feral swine living in regions where HPAIV H5N1 was diagnosed in wild birds should be continued. We believe more attention should be given to feral swine in influenza surveillance and monitoring because of the role it may play in the circulation and evolution of influenza.

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