ORIGINAL ARTICLE

Detection of Haemagglutination–Inhibiting Antibodies against Human H1 and H3 Strains of Influenza A Viruses in Pigs in Ibadan, Nigeria

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Impacts

- Human-type Influenza viruses [A/Brisbane/59/2007 (H1N1) and A/Brisbane/10/2007 (H3N2)] infect pigs in Ibadan, Nigeria.
- The level of transmission of influenza A/Brisbane/59/2007 (H1N1) from humans to pigs in Ibadan, Nigeria, is higher than that of influenza A/Brisbane/10/2007 (H3N2).
- The epizootiology of swine influenza in Ibadan may be influenced by currently circulating human influenza virus strains.

Keywords:
HI antibodies; human strains; influenza A viruses; pigs; Ibadan

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Summary

Agricultural and commercial activities have continued to bring people and pigs into regular, close contact in Ibadan, Nigeria. This study was therefore designed to investigate the transmission of human influenza viruses to pigs in Ibadan, using serological surveillance. Serum specimens were collected from ninety-one (91/199) apparently healthy, unvaccinated Landrace pigs at three locations within Ibadan from April to June, 2008. Two strains of human influenza virus A: A/Brisbane/59/2007 (H1N1) and A/Brisbane/10/2007 (H3N2) were used in Haemagglutination-Inhibition Assay for antibody detection. Prevalence of HI antibodies to the two subtypes was 90.1%. Antibodies to influenza A/Brisbane/59/2007 (H1N1) were significantly ($P < 0.05$) more prevalent (80.2%) than those of influenza A/Brisbane/10/2007 (H3N2) (51.6%). Titres of HI antibodies to influenza A/Brisbane/59/2007 [mean = 3331.5] were significantly higher ($P < 0.05$) than those of influenza A/Brisbane/10/2007 [mean = 2212.3]. This study shows that these pigs were exposed to human strains of influenza A(H1N1) and A(H3N2) either prior to or during this study. The implications of these high prevalence and antibody titres are discussed in relation to influenza virus infection among pig handlers in Ibadan, Nigeria. We recommend that periodic investigation of circulating strains of influenza viruses in pigs and humans who handle pigs regularly in Nigeria and molecular characterization of such isolates be carried out to ensure early detection of interspecies transmission and potential future pandemic strains.

Introduction

The success of interspecies transmission of influenza viruses depends on the viral gene constellation. Successful transmission between species can follow genetic reassortment, with a progeny virus containing a specific gene constellation having the ability to replicate in the new host (Webster et al., 1992; Webster and Hulse, 2004). Influenza A viruses constitute one of the five genera in the family *Orthomyxoviridae* (Kawaoka et al., 2005). They possess an eight-segment, negative-sense, ssRNA genome (Palese and Shaw, 2007). Based on the antigenicity of their two membrane glycoproteins, influenza A viruses are further subdivided into sixteen Haemagglutinin (H1-H16) and nine Neuraminidase (N1-N9) subtypes (Fouchier et al., 2005; Wright et al., 2007). Pigs can be

naturally or experimentally infected with avian and human influenza viruses because the epithelial cells in pig trachea contain both NeuAc-2,3Gal and NeuAc-2,6Gal receptors (Ito et al., 1996; Zhou et al., 1999) required by avian and human influenza viruses respectively (Castrucci et al., 1993; Kida et al., 1994). Pigs also appear to have a relatively weak species-specific barrier against infection by avian and human influenza A viruses (Scholtissek et al., 1993). Thus, classical swine viruses and avian-human reassortant viruses from pigs can infect humans and, as reported in some cases, cause fatal disease (Hinshaw et al., 1978; Claas et al., 1994). These observations support the 'mixing vessel' hypothesis that pigs, when simultaneously infected with avian and human influenza viruses, permit the generation of reassortants capable of causing pandemics (Scholtissek et al., 1985).

The focus of such reassortment has historically been in Southeast Asia, the proposed 'influenza epicenter,' because agricultural practices in this region brought pigs, people and ducks into close contact with one another (Webster et al., 1992). However, it is now clear that influenza virus reassortment in pigs can occur anywhere in the world, as evidenced by reassortant viruses isolated from pigs in Europe (Campitelli et al., 1997; Schrader and Süss, 2004) and in the United States (Webby et al., 2000). Pigs infected with human H1N1 or H3N2 influenza virus readily develop specific antibodies to these viruses (Brown et al., 1995a). Agricultural and commercial activities have continued to bring people and animals into regular, close contact in Ibadan, Nigeria. This study was therefore designed to investigate the transmission of human influenza viruses to pigs in Ibadan, using serological surveillance.

Materials and Methods

Sampling method and specimen collection

Using stratified random sampling, serum specimens were collected from ninety-one out of one hundred and ninety-nine (91/199) apparently healthy Landrace pigs at three locations within Ibadan, Oyo State, Nigeria from April to June, 2008 and tested at the Department of Virology, College of Medicine, University of Ibadan (U.I). These animals included boars (24 out of 76 available boars), sows (44/64), and growers (23/59). The locations are: Commercial Pig Farm Unit, U.I (11 out of 31 available pigs); University Research Farm, U.I (10/35) and Municipal Abattoir, Bodija (70/133). The history of these pigs showed that they were obtained from different sources and they had not been administered with influenza vaccine.

Pigs sampled on farms were bled through the cranial venacava, while blood sample was collected from the jugular vein immediately after slaughter at the abattoir. About 5 ml of blood was collected from each pig into labelled sample bottles (without anticoagulants) and allowed to clot. These were centrifuged in the laboratory at 3000 rpm for 10 min. Sera were then removed using Pasteur pipettes and stored in labelled eppendorf tubes at −20°C till they were tested.

Influenza Haemagglutination-Inhibiting (HI) antibody detection

Haemagglutination Inhibition Assay was used for antibody detection. This was performed according to World Health Organization (WHO) Protocol for Animal Influenza Diagnoses and Surveillance Manual (WHO, 2002). The virus strains used are influenza A/Brisbane/59/2007 (H1N1) and A/Brisbane/10/2007 (H3N2) CDC reference antigens. These consist of egg-grown viruses which had been concentrated, partially purified and inactivated by treatment with betapropiolactone (WHO, 2008). Sheep Influenza A/Brisbane/59/2007 (H1N1) (Homologous HI titre 1024/4HA) and A/Brisbane/10/2007 (H3N2) (Homologous HI titre 256/4HA) CDC reference antisera were used as positive serum controls. Non-specific inhibitors of haemagglutination were removed from all test sera and positive serum controls by receptor destroying enzyme (RDE) treatment, to obtain a 1 : 10 dilution which had one volume serum, three volumes RDE and six volumes physiological saline.

The HI procedure was as follows: Dilutions which contained 4HA units/25 μl of reference antigens were obtained before each test and a back-titration of the 4HA was performed to verify its correctness. Dilution (1 : 10) of each test serum and control serum was then prepared through RDE treatment. Two rows of wells in a V-bottom microtitre plate were labelled for each test and control serum and 25 μl of PBS was added to wells 2 through 12 of each row. Fifty microlitres of each treated serum was then added to the first well labelled for it, from where 25 μl was two-fold serially diluted across the row and discarded after well 10 to give a dilution of 1 : 20 through 1 : 5120. The last two columns were used as red blood cell (RBC) control wells. Twenty-five microlitres of standardized reference antigens was then added to the appropriate wells. Plates were agitated manually and incubated at room temperature for 15 min, after which 25 μl of 0.5% suspension of chicken RBCs was added to all wells. Plates were manually agitated and incubated at room temperature (25°C) for 30 min.

Interpretation of results

Endpoints of serum dilutions which showed complete inhibition of haemagglutination of 4HA units of the virus

were determined and the reciprocal of the endpoint of such serum specimens were recorded as the HI titre. Results obtained were analysed by two-way ANOVA and Student's *t*-test, using GRAPHPAD PRISM (GraphPad Software Inc., San Diego, CA, USA). Values of $P < 0.05$ were considered significant.

Results

Overall prevalence of HI antibodies to the two human strains of influenza viruses used during this study was 90.1% ($n = 91$). There was no significant difference ($P > 0.05$) in the values obtained among categories of pigs and locations and between months of sampling. However, the prevalence of HI antibodies to the two strains varied significantly ($P < 0.05$) within each category, location and month of sampling. Antibodies to influenza A/Brisbane/59/2007 (H1N1) were significantly ($P < 0.05$) more prevalent (80.2%) than those of influenza A/Brisbane/10/2007 (H3N2) (51.6%). These results are shown in Tables 1 and 2 respectively. Titres of HI antibodies to influenza A/Brisbane/59/2007 (H1N1) [mean = 3331.5] was significantly higher ($P < 0.05$) than those of influenza A/Brisbane/10/2007 (H3N2) [mean = 2212.3]. These titres are shown in Table 3. Percentages of pigs which had HI antibody titre greater than 20 HIU/25 μl to the strains tested are also shown in Table 3. Moreover, while 9 (9.9%) pigs had no detectable antibody level to either of the two strains, 41 (45.1%) reacted with only one influenza virus, while 41 (45.1%) also had polytypic reactions.

Table 1. Prevalence of HI antibodies to human influenza viruses in pigs at different locations

	Number positive (%)		
Location	Overall	A (H1N1)	A (H3N2)
Commercial Pig Farm Unit, U.I	11 (100.0)	11 (100.0)	0 (0.0)
University Research Farm, U.I.	8 (80.0)	7 (70.0)	3 (30.0)
Municipal Abattoir, Bodija, Ibadan.	63 (90.0)	55 (78.6)	44 (62.9)
Total	82 (90.1)	73 (80.2)	47 (51.6)

Table 2. Prevalence of HI antibodies to human influenza viruses in different categories of pigs

	Number positive (%)		
Category	Overall	A (H1N1)	A (H3N2)
Boar	24 (100.0)	20 (83.3)	12 (50.0)
Sow	38 (86.4)	36 (81.8)	23 (52.3)
Growwrs	20 (86.9)	17 (73.9)	12 (52.2)
Total	82 (90.1)	73 (80.2)	47 (51.6)

Discussion and Conclusion

The first report of interspecies transmission of influenza viruses involving pigs dates back to 1938, when Shope presented serological evidence for the transmission of a human influenza virus to pigs (Shope, 1936). Since then, other reports have described the transmission of human influenza viruses to pigs (Castrucci et al., 1994; Brown et al., 1998; WHO, 2004). In 1998, H3N2 viruses from humans were introduced into the pig population and caused widespread disease among pigs (Centre for Disease Control and Prevention, 2005).

Indeed, the possibility of human-to-swine transmission of influenza A viruses has been strengthened by numerous reports on the finding of antibodies to human strains of influenza virus type A, subtypes H1N1 and H3N2, in sera of pigs in different parts of the world. For instance, pigs in Germany had prevalence of 10.6%, 14.4% and 22.6% to A/Philippines/2/82 (H3N2), A/Hongkong/1/68 (H3N2) and A/Victoria/1/75 (H3N2) respectively, and two groups had 1.6% and 3.0% to A/Singapore/6/86 (H1N1) (Zhang et al., 1989); 57% and 62% to H3N2 in fattening and breeding pigs respectively (Teuffert et al., 1991); and 20.6% and 5.1% to A/Philippines/2/82 (H3N2) and A/Port Chalmers/1/73 (H3N2) (Ewald et al., 1994). Brown et al. (1995b) also described the findings of antibodies to a human strain of influenza virus A, subtype H3N2 (prevalence = 39%) in pigs in Great Britain, while a report of studies on wild pigs in Croatia gave prevalence of 18.81% to A/Johannesburg/82/96 (H1N1) and 87.12% to both A/Nanchang/933/95 (H3N2) and A/Wuhan/359/95 (H3N2) (Zupancic et al., 2000). In Japan, Shiraishi et al. (1989) reported the detection of antibodies to A/Shimane/1/80 (H3N2) (prevalence = 54.4%), while Katsuda et al. (1995) detected antibodies to human strains of influenza virus A, subtypes H1N1 and H3N2 in pigs. Other reports from Asia include that of a study on pigs in India, with prevalence of 43% and 46% to human strains of influenza virus A, subtypes H1N1 and H3N2 respectively (Chatterjee et al., 1995). Antibodies to A/Los Angeles/2/87 (H3N2) were also detected in pigs in the United States (Chambers et al., 1991). Apart from a few examples in which moderately high prevalence was reported, such as those of Shiraishi et al. (1989), Teuffert et al. (1991) and Zupancic et al. (2000), it could be observed that the prevalence of antibodies to the human strains of influenza A, subtypes H1N1 and H3N2, in the studies referred to above was generally low. However, previous serological studies on different swine herds in Nigeria, using human strains of influenza viruses, have shown that the prevalence tends towards very high values. Adeniji et al. (1993) reported prevalence of 74.6% and 86%

Table 3. Titres of HI antibodies to human influenza viruses in swine sera

Subtypes	Proportion positive	Number with HI-antibodies titres									Mean HI titre/25 μl	% with titres >20 HIU/25 μl
Titres (/25 μl)		10–20	40	80	160	320	640	1280	2560	5120		
A (H1N1)	73/91	N	N	04	06	04	04	07	06	42	3331.5	80.2
A (H3N2)	47/91	01	01	03	08	02	03	08	07	14	2212.3	50.5

N, no detectable antibody level to the strains tested.

to influenza A/Taiwan/1/86 (H1N1) and A/Shanghai/11/87 (H3N2) respectively, in sera of pigs in Ibadan, Nigeria collected between April, 1989 and December, 1990.

In another study on pigs in Ibadan, Nigeria, reported in 1990, prevalence rates of antibodies to four human strains of influenza viruses, A/Chile/1/83 (H1N1), A/Taiwan/1/86 (H1N1), A/Mississippi/1/85 (H3N2) and A/Victoria/3/75 (H3N2), were 87%, 79%, 86% and 94% respectively (Olaleye et al., 1990). Furthermore, Aiki-Raji et al. (2004) reported a prevalence of 94.39% for antibodies to a human strain of influenza H1N1in sera of pigs in Ibadan, Nigeria. In this study, the combined prevalence of 90.1% obtained for influenza viruses A/Brisbane/59/2007 (H1N1) and A/Brisbane/10/2007 (H3N2) follows this trend. When compared with some studies in which low prevalence were reported (such as Zhang et al., 1989 and Ewald et al., 1994; where 1268 and 2115 pigs were tested respectively), the high prevalence obtained in Nigeria (such as by Aiki-Raji et al., 2004; Adeniji et al., 1993; and in this study, where 107, 386, and 91 pigs were tested respectively) and in other parts of the world (such as by Zupancic et al., 2000, where 101 pigs were tested) could have been influenced by the relatively lower numbers of pigs sampled. However, as the history of the pigs in the present study clearly showed that influenza vaccines had not been administered and any maternally derived influenza antibodies must have waned considerably in these categories of pigs, our findings indicate that human-to-swine transmission of influenza viruses occurs in Ibadan, Nigeria. The great efficiency of this interspecies transmission could be attributed to some inadequacies associated with intensive swine husbandry system in Ibadan, Nigeria. These include lack of restriction of pig handlers to specific units of a farm, and sharing and rotation of farm workers among pig farms in the same area. For instance, 75% ($n = 16$) of pig handlers administered questionnaires in the two locations at the University of Ibadan had regular, close contact with pigs on both farms. Furthermore, we observed at the three locations that the levels of environmental and pig handlers' personal hygiene were generally low. These factors could facilitate transmission of influenza viruses from pig handlers to pigs, especially during the acute phase of influenza infections.

In addition, comparison of results obtained in the present study, in which antibodies to influenza A/Brisbane/59/2007 (H1N1) were significantly ($P < 0.05$) more prevalent (80.2%) than those of influenza A/Brisbane/10/2007 (H3N2) (51.6%), and results of similar previous studies in Ibadan, Nigeria, sited above, shows that while infection of swine populations in Ibadan with human strains of influenza virus A(H1N1) is still very high, infection of pigs with human strains of influenza virus A(H3N2) has reduced. This could be associated with changes in strains of circulating human influenza viruses among pig handlers in Ibadan. Further studies on this observation are on-going.

The HI test is considered a relatively sensitive test as the HA protein is quite antigenic and stimulates high circulating antibody concentrations. Titres of 1 : 40 or less may include non-specific reactions, but titres of 1 : 80 and above are considered positive and specific (Janke, 2000). However, the limit of HI positivity has been set at various titres by different authors. These include HI titre/25 μl: ≥1 : 8 (Katsuda et al., 1995); ≥1 : 20 (Dedek et al., 1990; Youzbashi et al., 1996; Zupancic et al., 2000); ≥1 : 32 (Kawano et al., 1978); and ≥1 : 40 (Shiraishi et al., 1989; Chambers et al., 1991; Markowska-Daniel and Pejsak, 1999). In this study, we took titres ≥1 : 10 HIU/25 μl as positive. Nevertheless, while 80.2% of the 91 pigs had titres ≥1 : 80 (mean = 3331.5 HIU/25 μl) to influenza A/Brisbane/59/2007 (H1N1), only 49.5% had titres ≥1 : 80 (mean = 2212.3 HIU/25 μl) to influenza A/Brisbane/10/2007 (H3N2). Using 0.5% chicken RBC, these high titres indicate recent infection. It is also important to note that, in spite of the high antibody titres, all pigs tested were subclinically infected. This could indicate that these pigs had been previously exposed to similar strains of influenza viruses, and that these responses are actually anamnestic (Wright et al., 1983, 2007).

We also found that prevalence of antibodies to the two human strains tested in this study differed significantly. This is different from the results of a previous study (Olaleye et al., 1990) in which no significant difference was observed between prevalence of antibodies to four strains of influenza A viruses in pigs in Ibadan, Nigeria.

In this study, we have provided serological evidence of human-to-swine transmission of influenza A viruses in Ibadan, Nigeria. Thus, the possibility of genetic reassortment between classical swine- and human influenza viruses in these pigs, leading to generation of novel influenza viruses with pandemic potentials is high. We therefore recommend that periodic investigation of circulating strains of influenza viruses in pigs and humans who handle pigs regularly in Nigeria, and molecular characterization of such isolates be carried out to ensure early detection of interspecies transmission and potential future pandemic strains.

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