

# Isolation of influenza A viruses from pigs in Ibadan, Nigeria

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## Summary

The authors investigated influenza virus types in pigs in Ibadan, a city in Oyo State, south-western Nigeria. From April to June 2008, nasal swabs were collected from pigs at three locations in Ibadan. Influenza A viruses (four A [H1N1], two A [H3N2] and one A [H1N1]-A [H3N2] double reactant) were isolated from 7 of the 50 apparently healthy Landrace pigs tested. This study is the first documented isolation of swine influenza viruses in Nigeria and it reveals that different strains of influenza viruses co-circulate in pigs in Ibadan. These pigs serve as reservoirs of different subtypes of influenza viruses and potential 'mixing vessels' in which genetic reassortment and the generation of future human pandemic strains could occur. These findings support the need for adequate and periodic surveillance among swine populations and pig handlers in Nigeria to detect circulating strains of influenza viruses. This would serve as an effective early warning system.

## Keywords

Flu, Ibadan, Influenza A, Isolation, Nigeria, Pig, Swine, Virus.

## Isolamento dei virus dell'influenza A in suini di Ibadan, Nigeria

### Riassunto

*In questo lavoro sono stati studiati i tipi di virus influenzali presenti nei suini della città di Ibadan, Stato di Oyo, Nigeria sud occidentale. Tra aprile e giugno 2008, da suini di tre differenti aree di Ibadan sono stati raccolti tamponi nasali. I virus dell'influenza A (quattro A [H1N1], due A [H3N2] e uno A [H1N1]-A [H3N2] a doppia reazione) sono stati isolati in 7 dei 50 suini di razza Landrace apparentemente sani. Lo studio, il primo documentato sull'isolamento dei virus dell'influenza suina in Nigeria, ha dimostrato la co-presenza di diversi ceppi nella popolazione suina di Ibadan. I suini fungerebbero da riserva per differenti sottotipi di virus dell'influenza e da potenziali "vasi di miscelazione" in cui potrebbero aver luogo il riassortimento genetico e la generazione di ceppi futuri, possibile causa di pandemia umana. I dati emersi confermano la necessità di un adeguato monitoraggio periodico delle popolazioni suine e degli allevatori in Nigeria per rilevare la presenza di ceppi di virus influenzali da impiegare come efficace sistema di pre-allerta.*

### Parole chiave

Ibadan, Influenza A, Isolamento, Maiale, Nigeria, Suino, Virus.

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## Introduction

Influenza A viruses constitute one of the five genera in the family *Orthomyxoviridae* (15). They possess an eight-segment, negative-sense, ssRNA genome which is approximately 13 kb in size (20, 26). 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 (11). Four main influenza A virus subtypes have been isolated in pigs. These are: H1N1, H1N2, H3N2 and H3N1 (8). These comprise the following:

- the H1N1 that are descendants of the H1N1 1918/1919 isolates (classical swine H1N1)
- the H1N1 that are similar to viruses isolated from avian species (avian-like H1N1 swine influenza viruses)
- H3N2 viruses that are antigenically or genetically similar to those isolated from humans (human-like swine influenza viruses) (27).

A number of findings have suggested a role for pigs in the emergence of pandemic influenza viruses. To start with, 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 (13, 30) that are required by avian and human influenza viruses, respectively, to initiate infection (7, 16). Secondly, in nature, continued replication of an avian influenza virus in pigs leads to variants that preferentially recognise human-type receptors (21). Thus, classical swine viruses and avian-human reassortant viruses from pigs can infect humans and, as reported in some cases, can cause a fatal disease (9, 12). These observations support the 'mixing vessel' hypothesis that pigs, when simultaneously infected with avian and human influenza viruses, enable the generation of reassortants capable of causing pandemics (22).

Pigs also appear to have a relatively weak species-specific barrier against infection by avian and human influenza A viruses (23). Furthermore, there is evidence that some strains of influenza viruses persist in pigs many years after they have disappeared from

the human population (27). This makes the pig an important reservoir of the virus from which a susceptible human population could later be infected. There is a dearth of information on the types of swine influenza viruses circulating in Nigeria. This study was therefore designed to investigate the types and subtypes of influenza viruses currently circulating in pigs in Ibadan, Nigeria. This is important for early detection of reassortant swine influenza viruses which could have pandemic potential.

## Materials and methods

### Sampling method and specimen collection

There were 601 registered pig farmers on the list of the Pig Farmers' Association of Nigeria and the Ministry of Agriculture in Oyo State, Nigeria. These farmers had an estimated 63 832 pigs in January 2001, shortly before the first outbreak of African swine fever that led to the culling and mortality of many pigs in Oyo State (4). Using stratified random sampling, nasal swabs were collected from 50 out of 199 (50/199) apparently healthy Landrace pigs at three locations within Ibadan, a city in Oyo State, south-western Nigeria (Fig. 1) from April to June 2008 and tested at the Department of Virology, College of Medicine of the University of Ibadan. The locations can be listed as follows:

- commercial pig farm unit and slaughter house, University of Ibadan
- Teaching and Research Farm, University of Ibadan
- Municipal Abattoir, Bodija.

The categories of pigs tested are shown in Table I. Nasal swabs were obtained by inserting a sterile swab into the nares, allowing it to remain there for a few seconds and then slowly withdrawing it, using a rotating movement down the side of the nares. One swab was used for both nares. The swab was immediately inserted into a labelled 2 ml cryovial containing virus transport medium. This was screw-capped after the applicator stick had been broken off. Virus transport medium, maintained at pH 7.2-7.4, was prepared by adding 15 ml penicillin-streptomycin and

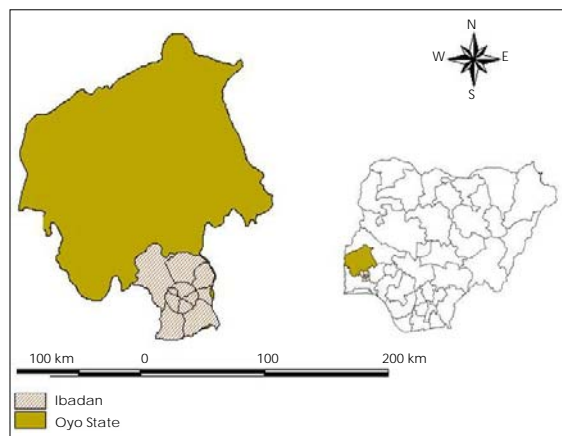


Figure 1  
Study area with an insert showing the location in south-western Nigeria

0.6 ml fungizone to 500 ml of the Dulbecco modified eagle medium (D-MEM) without foetal bovine serum (FBS) (i.e. 0% D-MEM). Specimens in transport medium were transferred immediately into ice-packs before transporting to the laboratory for virus isolation, or for temporary storage at  $-80^{\circ}\text{C}$ .

Table I  
Categories of pigs tested in the study

Category	Proportion tested
Boars	19/76
Sows	16/64
Growers	15/59
Total	50/199

### Virus isolation

Inoculation and harvesting of cells were performed in a class 2 biosafety cabinet. Each freshly collected or stored specimen (0.2 ml) was inoculated in duplicate into appropriately labelled tubes with confluent monolayer of human epithelium-2 (HEp-2) cell line in growth medium (10% D-MEM, i.e. containing 450 ml D-MEM and 50 ml FBS) using sterile 1 ml pipettes. A HEp-2 cell line was used because of a major delay in obtaining Madin-Darby canine kidney (MDCK) cell lines, which has been shown to be quite sensitive, especially for detecting mammalian influenza virus isolates (5, 17). Those stored at  $-80^{\circ}\text{C}$  were first thawed on ice. Tubes were properly arranged in racks, incubated at  $37^{\circ}\text{C}$  and

observed daily for cytopathic effects (CPE) for 7 days. Cell cultures which revealed CPE were observed and harvested when the level of CPE had attained 75% and above, to a maximum of 100% (i.e. 3+ to 4+). Supernatant from such tubes were stored as aliquots of 2 ml at  $-80^{\circ}\text{C}$  for subsequent passaging. Aliquots for blind passaging were also taken from cultures without CPE by days 6 or 7 post inoculation. Supernatant fluids were passaged twice. Those without CPE during the second passage were considered negative.

The steps involved in isolation in embryonated eggs were also performed in a class 2 biosafety cabinet. Ten to twelve day-old embryonated chicken eggs were candled with the blunt end up and viable eggs were selected. These were not specific-pathogen-free eggs, but were obtained from breeder stocks that had never been vaccinated against influenza and which were raised on farms on which avian influenza had not been reported. Furthermore, two viable, uninoculated, 10-12 day-old embryonated chicken eggs obtained from the same source and batch as the inoculated eggs were included with each batch of inoculated eggs as negative controls. The limits of the air sacs were marked. The surface of each egg was disinfected and 200  $\mu\text{l}$  of specimen was inoculated through a hole drilled on the blunt end, into the allantoic cavity using a 2 ml syringe with 23G needles. Each specimen was inoculated in duplicate. After sealing these holes with paraffin wax, the eggs were labelled and incubated at  $37^{\circ}\text{C}$ . Incubated eggs were observed daily for embryonic death. Eggs in which death occurred from 24 h post inoculation were immediately chilled at  $4^{\circ}\text{C}$  overnight or for 4 h, before harvesting to congeal the blood in order to prevent haemorrhaging, while those without embryonic death were incubated for 72 h to 96 h before chilling and harvesting. After aseptic preparations, the allantoic fluids of the eggs were harvested into 5 ml cryo-vials. Aliquots were made in duplicate from haemagglutination assay (HA)-positive specimens. HA was performed according to World Health Organization (WHO) *Manual on animal influenza diagnosis and surveillance* (27). One of

these was stored at  $-80^{\circ}\text{C}$  for further identification, while the other was used for passaging in a new batch of eggs. Specimens which were HA-negative initially were also passed into new batches of eggs and retested before being considered negative.

### Identification of isolates

The haemagglutination inhibition assay (HI) was used for isolate identification. This was performed according to the *Manual on animal influenza diagnosis and surveillance* (27). The antisera used were as follows:

- sheep influenza A (H1N1), Centers for Disease Control and Prevention (CDC) reference antiserum
- sheep influenza A (H3N2), CDC reference antiserum
- sheep influenza B/Shanghai/361/2002-like (B/Yamagata/16/88 lineage), CDC reference antiserum
- sheep influenza B/Malaysia/2506/2004-like (B/Victoria/2/87 lineage), CDC reference antiserum.

The control antigens were as follows:

- influenza A (H1N1), CDC reference antigen
- influenza A (H3N2), CDC reference antigen
- influenza B/Shanghai/361/2002-like (B/Yamagata/16/88 lineage)
- influenza B/Malaysia/2506/2004-like (B/Victoria/2/87 lineage) (28).

We restricted our tests to the above influenza virus antisera and antigens because of difficulties encountered in obtaining a complete influenza A virus identification panel. Non-specific inhibitors of haemagglutination were removed from the reference antisera and test sera by receptor destroying enzyme (RDE) treatment. This was performed by adding 3 volumes of RDE to 1 volume of serum and incubating the mixture in a water bath at  $37^{\circ}\text{C}$  overnight or for 12-18 h. This was then heated in a water bath at  $56^{\circ}\text{C}$  for 30 min. After the mixture had been allowed to cool to room temperature, 6 volumes of physiological saline (0.85% NaCl) were added to obtain a 1:10 dilution which had 1 volume serum, 3 volumes RDE and 6 volumes physiological saline (27).

The HI procedure was conducted as follows: dilutions which contained 4 HA units/25  $\mu\text{l}$  of isolate or control antigens against 0.5% chicken red blood cell (RBC) suspension were obtained before each test and a back-titration of the 4 HA was performed to verify its accuracy. Dilution (1:10) of each antiserum (or test serum) was then prepared through RDE treatment. Two rows of wells in a V-bottom microtitre plate were labelled for each isolate or control antigen and 25  $\mu\text{l}$  of phosphate buffered saline (PBS) were added to wells 2 to 12 of each row. A total of 50  $\mu\text{l}$  of each treated serum were then added to the first well thus labelled and from which 25  $\mu\text{l}$  were serially diluted with a dilution factor of 1/2 across the row and discarded after well 10 to give a dilution of 1:20 through 1:5 120. The last two columns were used as RBC control wells. A total of 25  $\mu\text{l}$  of standardised antigens and field isolates were then added to the appropriate wells. Plates were agitated manually and incubated at room temperature for 15 min, after which 25  $\mu\text{l}$  of 0.5% chicken RBC suspension was added to all wells. Plates were manually agitated and incubated at room temperature ( $25^{\circ}\text{C}$ ) for 30 min. Results were then read and recorded.

### Interpretation of results

An isolate was identified as a particular type or subtype if it clearly reacted with only one antiserum or, in cases of cross-reactivity, if it reacted with one antiserum giving an HI titre that was four-fold or more than its titres to the other antisera (27).

## Results

The CPE was characterised by cell rounding, degeneration and aggregation of cells into grape-like clusters. Of the 50 swine specimens collected for virus isolation, 7 (14.0%) were positive by HI. The seven isolates were distributed as follows:

- four influenza A (H1N1)
- two influenza A (H3)
- one influenza A (H1N1)-A (H3N2) double reactant.

Results of virus isolation based on category shows that 20%, 12.5% and 10.5% of the

growers, sows and boars sampled, respectively, had influenza virus infection. Table II shows that isolation in 10 to 12 day-old embryonated eggs was more sensitive (detection rate = 14.0%) than isolation in HEp-2 cell line (detection rate = 0%). Embryos in all uninoculated eggs (negative controls) remained viable. Samples which showed CPE in HEp-2 were passed into MDCK. Of 4 samples, only one displayed CPE in MDCK. This sample had HA activity but did not react with any of the four reference antisera. The HI titres of swine isolates obtained in this study and control antigens used are given in Tables III and IV.

## Discussion and conclusion

Serological surveillance studies revealed that prevailing human H1N1 strains are readily transmitted to pigs (6). Human influenza-like A (H3N2) viruses have also been isolated from pigs (24, 30), but are not usually maintained in pigs independently of the human population (14). Influenza occurs most often in Nigeria during the harmattan period (November to January) and early (April to May) to the peak rainy season (10, 18). Previous studies have shown that the prevalence of HI antibodies to different influenza viruses is generally high in humans, swine and poultry in Nigeria (1, 18,

Table II  
Results of virus isolation in different culture systems

Culture system	Cytopathic effect/ embryonic death	Number positive (%) Haemagglutination assay result	Haemagglutination inhibition assay result
Embryonated eggs	46(92.0)	13(26.0)	7(14.0)
HEp-2	4(8.0)	1(2.0)	0(0.0)

HEp-2 human epithelium-2

Table III  
Titres of positive controls in haemagglutination and haemagglutination inhibition assays

Reference antigen	Haemagglutination titre (/25 µl)	Haemagglutination inhibition titre(/25 µl) with reference antisera			
		A (H1N1)	A (H3N2)	B/Shanghai	B/Malaysia
A (H1N1)	64	160	-	-	-
A (H3N2)	32	-	20	-	-
B/Shanghai	128	-	-	80	-
B/Malaysia	64	-	-	-	20

Table IV  
Titres of swine influenza virus isolates in haemagglutination inhibition assay

Serial No.	Field isolate identification number	Haemagglutination inhibition titres/25 µl of field isolates from two culture systems							
		Embryonated eggs				HEp-2 cell line			
		A (H1N1)	A (H3N2)	B/S	B/M	H1N1	H3N2	B/S	B/M
1	IB/FLU 08/P002	+(40)	-	-	-	-	-	-	-
2	IB/Flu 08/P003	+(40)	+(40)	-	-	-	-	-	-
3	IB/Flu 08/P005	-	+(40)	-	-	-	-	-	-
4	IB/Flu 08/P012	+(160)	-	-	-	-	-	-	-
5	IB/Flu 08/P015	+(320)	-	-	-	-	-	-	-
6	IB/Flu 08/P017	-	+(20)	-	-	-	-	-	-
7	IB/Flu 08/P041	+(20)	-	-	-	-	-	-	-

HEp-2 human epithelium-2

IB influenza B

B/S influenza B Shanghai/361/2002-like

B/M influenza B Malaysia/2506/2004-like



19). In the present study, which is the first documented isolation of swine influenza viruses in Nigeria, investigation using samples collected within one of the two peak periods of influenza infection (April to June 2008), revealed that two subtypes of influenza A viruses, H1 and H3, co-circulated among apparently healthy pigs in Ibadan during this period. Reactivity of one of the isolates obtained with two different reference antisera, giving equal HI titres, could indicate the presence of epitopes of the HA of the cross-reacting viruses in these field isolates. This could be due to co-infection by these viruses, and, as this is a preliminary report, we hope to conduct further studies to verify this possibility.

Results from a recent study by the authors also revealed that different strains of influenza viruses currently circulate among pig handlers in Ibadan (2). While attempts are being made to perform molecular characterisation of these swine and human influenza isolates, the possibility of interspecies transmission, especially from pig handlers to these pigs, is enormous. This is because pigs appear to have a relatively weak species-specific barrier against infection by avian and human influenza A viruses (23) and pigs in Ibadan were found to have high prevalence of HI antibodies to human influenza viruses (3).

Since influenza viruses with a potential to become pandemic are generated through antigenic shift which is thought to occur through one of three mechanisms, namely:

- direct transfer of whole virus from another species

- genetic reassortment of avian and human influenza A viruses infecting the same host
- re-emergence of a virus that may have caused an epidemic many years earlier (25, 29).

The co-circulating strains of human and swine (and possibly avian) influenza viruses may therefore combine in these pigs to form novel, reassortant viruses which could be highly virulent when established in human, swine or avian populations. This possibility portends sinister prospects, especially when considered along with the observation that two of the pig farms sampled for this study were located within a 5-10 km radius of commercial poultry farms. Periodic surveillance should therefore be conducted to detect the types and subtypes and strains of influenza viruses in pigs in Nigeria. The surveillance of influenza in pigs is useful to acquire knowledge of circulating viruses as well as for vaccination programmes. Moreover, it is an important tool for monitoring the generation of new strains of human influenza which could have a pandemic potential.

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