# Evidence for Subclinical H5N1 Avian Influenza Infections Among Nigerian Poultry Workers

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In recent years Nigeria has experienced sporadic incursions of highly pathogenic H5N1 avian influenza among poultry. In 2008, 316 poultry-exposed agricultural workers, and 54 age-group matched non-poultry exposed adults living in the Enugu or Ebonyi States of Nigeria were enrolled and then contacted monthly for 24 months to identify acute influenza-like-illnesses. Annual follow-up sera and questionnaire data were collected at 12 and 24 months. Participants reporting influenza-like illness completed additional questionnaires, and provided nasal and pharyngeal swabs and acute and convalescent sera. Swab and sera specimens were studied for evidence of influenza A virus infection. Sera were examined for elevated antibodies against 12 avian influenza viruses by microneutralization and 3 human viruses by hemagglutination inhibition. Four (3.2%) of the 124 acute influenza-like-illness investigations yielded molecular evidence of influenza, but virus could not be cultured. Serial serum samples from five poultry-exposed subjects had a ≥4-fold change in microneutralization titers against A/CK/Nigeria/07/1132123(H5N1), with three of those having titers ≥1:80 (maximum 1:1,280). Three of the five subjects (60%) reported a preceding influenza-like illness. Hemagglutination inhibition titers were ≥4-fold increases against one of the human viruses in 260 participants. While cross-reactivity from antibodies against other influenza viruses cannot be ruled out as a partial confounder, over the course of the 2-year follow-up, at least 3 of 316 (0.9%) poultry-exposed subjects had evidence for subclinical HPAI H5N1 infections. If these data represent true infections, it seems imperative to increase monitoring for avian influenza among Nigeria's poultry and poultry workers. ***J. Med. Virol.*** © 2014 Wiley Periodicals, Inc.

**KEY WORDS:** zoonoses; occupational exposure; communicable diseases, seroepidemiology

## INTRODUCTION

Since first detections of highly pathogenic avian influenza (HPAI) in 2006 [Fasina et al., 2011], the virus has caused occasional outbreaks among Nigeria's poultry flocks. As of December 2013, a total of 65 poultry HPAI subtype H5N1 outbreaks in Nigeria have been reported to the World Organization for Animal Health (OIE) since the end of 2003; the last avian infection on record from Nigeria occurred in September 2008, resulting from one of two outbreaks that year that caused a total of 1,545 cases. Since 2003, 641 human HPAI H5N1 infections have been reported globally by WHO; in 2007 the only reported human infection with HPAI H5N1 from Nigeria was documented. To understand the threat avian influenza viruses (AIVs) pose to persons exposed occupationally to poultry, a 2-year prospective cohort study of Nigerians ≥18 years was initiated. Throughout this prospective study, agricultural workers in Nigeria were enrolled where larger poultry

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production facilities have been introduced, yet open animal markets still thrive. It was hypothesized that poultry workers would have more evidence of zoonotic influenza virus infections compared to Nigerians not exposed to live poultry. Enrollment serological results published previously found little evidence of previous AIV infection among the exposed cohort [Okoye et al., 2013].

## MATERIAL AND METHODS

### Study Subjects

During the period December 2008 to April 2010, 316 poultry-exposed and 54 age-group matched non-poultry exposed participants (≥18 years) were enrolled from towns of Nsukka, Udi, and Enugu in Enugu State and the town Abakaliki in Ebonyi State, all in Southeast Nigeria (Fig. 1). These sites were selected for their proximity to the University of Nigeria where the study was based. Informed consent was obtained from all study participants. A total of four institutional review boards (University of Iowa, University of Florida, University of Nigeria, and Human Research Protection Office of the U.S. Army Medical Research and Materiel Command) reviewed and approved the study. All experiments were performed in compliance with relevant laws and institutional guidelines and in accordance with the ethical standards of the Declaration of Helsinki. Details of location, study participant demographics, enrollment methods, and serology laboratory methods have been published previously [Okoye et al., 2013]. The cohort was followed through December 2011 for evidence of influenza-like-illness. Sera and questionnaire data were collected at enrollment, 12 and 24 months. Questionnaires collected demographic, health, and animal exposure changes over the previous year. Poultry exposures were defined as ≥5 cumulative hours/week for at least 1 week.

### Monthly Follow-Up

Upon enrollment, participants were given oral and written instructions and a digital thermometer to help identify influenza-like symptoms. Influenza-like illness was defined as an acute onset of a respiratory illness with an oral (or equivalent from other body region) measured temperature ≥38°C, and a sore

Fig. 1. Map of the four enrollment sites in Enugu State and Ebonyi State, Nigeria.

throat or cough. Each month, study staff contacted the subjects via telephone to learn if they had experienced an influenza-like illness event. One staff member was assigned a small region with a manageable number of participants to contact monthly. If a participant experienced these symptoms between monthly contacts with study staff, the participant was instructed to inform a staff member.

### Influenza-Like Illness Investigation

When a participant reported an influenza-like illness, a staff member conducted a home visit within 24 hr to confirm that the criteria were met. Questionnaire data, an acute serum sample and two respiratory swabs (nasal and pharyngeal) were collected by the staff member. Staff members also collected a convalescent serum sample 60 days after the initial investigation. If the participant experienced a second distinct case of influenza-like illness after the 60-day period, a new investigation was initiated and treated as a separate event.

### Laboratory Methods

Sera and influenza-like illness respiratory swab aliquots were preserved at −80°C and transported on dry ice to the University of Florida for testing. Sera were screened for evidence of avian and human influenza virus infections using hemagglutination inhibition and microneutralization assays (Table I). Respiratory swabs were screened for molecular evidence of influenza A virus using real-time RT-PCR. Viral antigens and control antisera for HI assays were obtained from acknowledged collaborators from the Biodefense and Emerging Infections (BEI) Research Resources Repository (Manassas, VA) or through the Influenza Reagent Resource (IRR) program (Manassas, VA).

TABLE I. Viruses Used in Serological Studies

| **Avian viruses** |
|---|
| A/Migratory duck/Hong Kong MPS180/2003(H4N6) |
| A/CK/Nigeria/07/1132123(H5N1)[a] |
| A/Nopi/Minnesota/07/462960-2(H5N2) |
| A/Teal/Hong Kong/w312/97(H6N1) |
| A/WF/Hong Kong/Mpb127/2005(H7N7) |
| A/Migratory duck/Hong Kong/MP2553/04(H8N4) |
| A/Migratory duck/Hong Kong/MPD268/2007(H10N4) |
| A/Hong Kong/1073/1999(H9N2)[b,c] |
| A/Chicken/New Jersey/15906-9/96(H11N1) |
| A/DK/ALBERT60/76(H12N5) |
| **Human viruses** |
| A/Brisbane/59/2007(H1N1)[d] |
| A/Mexico/4108/2009(H1N1)[b,d] |
| A/Brisbane/10/2007(H3N2)[d] |

Unless otherwise indicated, serologic study was performed using the microneutralization assay.
[a]Highly pathogen virus.
[b]Similar to 2009 pandemic H1N1 virus.
[c]Virus of avian origin but cultured from a man.
[d]Virus studied with hemagglutination inhibition assay.

***Real-time RT-PCR influenza assay.*** Viral RNA was isolated from 140 μl of each swab specimen and processed using the Qiagen QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA) following a mini-spin protocol. Contaminants were washed away by two wash buffers and the RNA eluted in 50 μl of elution buffer. Specimens were screened for the presence of influenza A viral RNA using the CDC's Human Influenza Virus Real-Time RT-PCR Detection and Characterization Panel [Jernigan et al., 2011]. Specimens that were Real-Time RT-PCR positive for generic influenza type A were further evaluated with an additional Real-Time RT-PCR procedure specific for Avian H5, and human H1, and H3 subtypes, as well as 2009 pandemic H1. Respiratory swab samples that tested positive and suspected positive for influenza A but could not be subtyped were cultured in Madin–Darby canine kidney (MDCK) cells and passaged twice in an attempt to amplify the virus for further studies.

***Hemagglutination inhibition (HI) assay.*** A WHO-recommended HI assay [Kayali et al., 2008] was used to test for serum antibodies against human influenza A viruses. Influenza virus strains were grown in MDCK cells or fertilized eggs. Sera were pre-treated with receptor destroying enzyme and hemabsorbed with either guinea pig or turkey erythrocytes. Titer results were reported as the reciprocal of the highest dilution of serum that inhibited virus-induced hemagglutination of a 0.65% (guinea pig) or 0.50% (turkey) solution of erythrocytes as established previously [Kendal et al., 1982].

***Microneutralization assay.*** A WHO-recommended microneutralization assay adapted from a report by Rowe [Rowe et al., 1999; Gill et al., 2006; Myers et al., 2007] was used to detect human antibodies against AIVs. The viruses were grown in fertilized eggs. Sera were first screened at a dilution of 1:10. Positive specimens were then titrated out in duplicate by examining twofold serial dilutions from 1:10 to 1:1,280 in virus diluents [85.8% minimum essential medium (Invitrogen, Carlsbad, CA), 0.56% BSA, 25 mM HEPES buffer (Invitrogen), 100 mg/L streptomycin (Invitrogen), and 100,000 units/L penicillin (Invitrogen)]. Virus neutralization was then performed by adding 100 $TCID_{50}$ of virus to the sera. The Reed Muench method was used to determine the $TCID_{50}$/100 μl [Reed and Muench, 1938]. MDCK cells in log phase growth were adjusted to $2.0 \times 10^5$ cells/ml with virus diluent. One hundred microliters of this suspension of cells were added to each well and the plates incubated at 37°C with 5% $CO_2$ for 24 hr. Plates were washed twice with PBS, fixed with cold 80% acetone, and incubated at room temperature for 10 min. Influenza on the fixed monolayers was then quantified by influenza A nucleoprotein-specific indirect ELISA. The plates were washed with phosphate buffered saline containing 0.05% Tween 20 between each antibody addition after 1 hr incubation at room temperature. Following the final wash, 0.1 ml of

3,3′,5,5′-tetramethylbenzidine (TMB) (KPL 50–76–03) (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was added and incubated at room temperature for 10 min. Color development was stopped by the addition of 0.1 ml of 1 M sulfuric acid. The optical density of each plate was read at 450 nm. The viral-neutralization endpoint titer was expressed as the reciprocal of the highest dilution of serum with optical density (OD) less than X, where X = [(average OD of virus control wells) + (average OD of cell control wells)]/2. Test cells with an OD > 2 times the cell control OD mean were considered positive for virus growth. A back titration of the viral antigen was run in duplicate and only accepted when both replicates had matching results.

### Influenza Case Definition and Identification

Study outcomes included evidence of previous or acute influenza A virus infections. Acute influenza infection was defined as either (a) isolation of influenza virus from a respiratory specimen obtained when a patient had an influenza-like illness, (b) real time RT-PCR evidence of influenza from such specimens, or (c) a fourfold or greater rise in antibody titer against an influenza virus across annual follow-up sera. Because serologic responses to zoonotic influenza infections can wane rapidly [Buchy et al., 2010], as reported previously [Gray et al., 2008; Khuntirat et al., 2011; Blair et al., 2013; Khurelbaatar et al., 2013], this study chose a low threshold of antibody titer (≥1:10) as evidence of previous infection with an AIV strain. Because it is known that cross-reactions from vaccines or previous infection with human influenza viruses might confound AIV serology, reactivity to human influenza strains were examined as evidence of such potential confounding. The prevalence of elevated titers for various influenza strains were computed using SAS v9.2 (SAS Institute, Cary, NC).

## RESULTS

Demographic and serology data collected from enrollment in 2008 and 2009 from 370 participants have been reported previously [Okoye et al., 2013]. Over the 24-month follow-up period no subjects were lost to follow-up and all study subjects participated in the 12- and 24-month follow-up encounters. This was accomplished through the use of incentives and diligent field work by study staff.

### Influenza Investigations

A total of 124 influenza-like illness investigations were performed during the 24 months of follow-up. Two subjects had multiple events (two each). Respiratory swab specimens were screened for influenza A by real time RT-PCR. Four specimens were found to be weakly positive, but no virus was cultured or identified molecularly. A total of 36 (29.0%) paired serum samples demonstrated a fourfold rise or greater in antibody titer [26 A/Brisbane/10/2007(H3N2), 7 A/Mexico/4108/2009(H1N1) a (H1N1)pmd09-like virus, 2 A/Brisbane/59/2007(H1N1), and 1 HPAI A/CK/Nigeria/07/1132123(H5N1)].

A subject (ID# 004) with a reported influenza-like illness and a rise in H5N1 serum titer (acute vs. convalenscent) had no elevated H5N1 titer upon enrollment or at the 12- and 24-month follow-ups. While ID# 004 self-reported exposure to pigs and chickens, the magnitude of the convalescent titer against H5N1 was low at 1:20; a very modest fourfold increase from the acute sample (≤1:10).

### Study of Annual Sera

Annual samples collected from all participants were compared over the 2 years of study (Tables II and III). While microneutralization titers against the other AIVs were sparse, five poultry-exposed subjects experienced fourfold or greater increases in titer against HPAI H5N1 (titers ranged from 1:20 to 1:1,280). Among these five, only two (ID# 065 and 128) reported an influenza-like illness that led to an investigation. Another participant (ID# 091) indicated experiencing a respiratory illness within the past year on the 24-month follow-up questionnaire. Medical history also revealed ID# 091 was a smoker and on pain medication, and exposure history revealed daily occupational poultry exposure. The 24-month serum sample revealed a high microneutralization titer (1:320) against H5N1. Subjects ID# 065 and ID# 128 had elevated titers at the 24-month follow-up, but results from the influenza-like illness specimens were negative. Both worked with chickens (ID# 065 also worked with turkeys) for 6 hr/day, but neither reported outbreaks at work or at home. The last two (ID# 099 and 114) did not report illness, but had daily poultry exposure at work exceeding 5 hr/day. Neither participant reported an illness outbreak among humans or animals at work. Subject ID# 114 reported his associates had respiratory illnesses, but denied any personal or family illness (24-month titer

TABLE II. Summary of Elevated Microneutralization Assay Results (≥1:10) Among 370 Nigerian Study Participants 2008–2011

| | 0-Month | 12-Month | 24-Month |
|---|---|---|---|
| AvH4N6 | 0 | 0 | 4 (1.1%)[a] |
| AvH5N1 HPAI | 1 (0.3%) | 4 (1.3%) | 5 (1.4%) |
| AvH6N1 | 0 | 1 (0.3%)[a] | 0 |
| AvH7N7 | 0 | 0 | 0 |
| AvH8N4 | 0 | 0 | 0 |
| AvH9N2 | 4 (1.1%) | 2 (1.3%)[a] | 0 |
| Av10N4 | 0 | 0 | 0 |
| AvH11N1 | 3 (0.8%) | 0 | 0 |
| AvH12N5 | 0 | 0 | 8 (2.2%) |
| HuH1N1 (Brisbane) | 66 (17.9%) | 77 (24.4%) | 40 (10.8%) |
| HuH3N2 (Brisbane) | 116 (31.4%) | 145 (49.5%) | 97 (26.6%) |
| A(H1N1)pmd09 | 6 (1.6%) | 18 (5.7%) | 51 (15.4%) |

[a]Assay results were generally of low titer or not sustained over time.

TABLE III. Select Study Subjects' Microneutralization Assay Results Examined Over Time, Nigeria 2009–2011

| Participant ID | 0-Month | 12-Month | 24-Month | Poultry exposed? |
|---|---|---|---|---|
| A/CK/Nigeria/07/1132123(H5N1) | | | | |
| 22 | <1:10 | 1:10 | <1:10 | Y |
| 65 | <1:10 | <1:10 | 1:20 | Y |
| 86 | <1:10 | 1:10 | <1:10 | Y |
| 91 | <1:10 | <1:10 | 1:320 | Y |
| 99 | <1:10 | 1:160 | <1:10 | Y |
| 103 | <1:10 | <1:10 | 1:10 | Y |
| 114 | <1:10 | <1:10 | 1:280 | Y |
| 128 | <1:10 | <1:10 | 1:40 | Y |
| 132 | <1:10 | 1:40 | <1:10 | Y |
| 357 | 1:80 | <1:10 | <1:10 | N |
| A/Nopi/Minnesota/07/462960-2(H5N2) | | | | |
| 22 | <1:10 | 1:10 | <1:10 | Y |
| 99 | 1:20 | <1:10 | <1:10 | Y |
| 102 | <1:10 | <1:10 | 1:20 | Y |
| 128 | <1:10 | 1:10 | <1:10 | Y |
| 132 | <1:10 | 1:10 | <1:10 | Y |
| 357 | 1:10 | <1:10 | <1:10 | N |
| A/Hong Kong/1073/1999(H9N2) | | | | |
| 24 | <1:10 | 1:20 | <1:10 | Y |
| 29 | 1:40 | 1:20 | <1:10 | Y |
| 95 | <1:10 | 1:20 | <1:10 | Y |
| 209 | 1:20 | <1:10 | <1:10 | Y |
| 227 | 1:20 | <1:10 | <1:10 | Y |
| 295 | <1:10 | 1:10 | <1:10 | Y |
| 300 | 1:20 | <1:10 | <1:10 | Y |
| A/Chicken/New Jersey/15906–9/96(H11N1) | | | | |
| 192 | 1:10 | <1:10 | <1:10 | Y |
| 209 | 1:40 | <1:10 | <1:10 | Y |
| 212 | 1:10 | <1:10 | <1:10 | Y |

1:1,280). The most compelling evidence of H5N1 infection seemed to be for subjects ID# 91, 99, and 114 with annual sera titer >1:80 (Table III). None of these subjects sought medical attention for influenza-like illness during the follow-up period so their HPAI H5N1 infections were likely either mild or asymptomatic.

Serial assays against the other AIVs (Tables II and III) were negative or of low titer and not sustained over time.

## DISCUSSION

There is considerable debate among influenza researchers regarding how to interpret serological assays against AIVs. Some embrace strict microneutralization titer criteria which requires a titer of 1:80 as evidence of HPAI H5N1 infection [WHO, 2007; Toner et al., 2013]. Others appreciate studies of mild HPAI H5N1 infections with titers declining rapidly, falling below 1:80 [Vong et al., 2009] and are a bit more inclusive [Morens, 2013]. Whether interpreted conservatively or not, the high microneutralization titers for several poultry workers in this study support the premise that Nigeria is continuing to experience H5N1 infections among poultry and these viruses are infecting Nigerian poultry workers occasionally.

This study had a number of limitations. It is likely that the collected specimens suffered from a faulty cold chain as influenza virus could not be cultured from the specimens, which were influenza-positive by molecular assays. It is quite possible with seroepidemiological studies of this kind that at least some AIV serological findings represent false positives due to cross-reacting antibodies from human influenza infections. The study focused upon persons ≥18 years of age and ignored children and the elderly. As has been suggested by other studies of AIV infections in man, both of these age groups may be at higher risk of AIV; therefore important at-risk sub-populations were excluded from this study. Additionally, due to a number of delays, including lengthy political demonstrations, the study controls were enrolled approximately 1 year after the exposed subjects, which could have introduced temporal exposure biases between the groups. Finally, the power supply in rural Nigeria is sporadic and the specimens' −80°C storage may have been compromised slightly with several freeze-thaw cycles before specimen shipment to the United States.

## CONCLUSIONS

In conclusion, while molecular or viral culture evidence of acute HPAI H5N1 infection among Nigerian poultry workers was not detected, the seroepidemiological study data suggest that some workers were exposed recently to H5N1 virus. Study data underscore the need for continued AIV surveillance among Nigeria's poultry and poultry workers.

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