

SEROLOGIC AND VIROLOGIC SURVEILLANCE OF AVIAN INFLUENZA IN NIGERIA, 2006-7

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Since January 2006, H5N1 avian influenza has affected Nigeria's poultry population causing enormous loss of resources. The current circulating virus is a potential candidate for pandemic influenza which may severely affect the human and animal population worldwide especially in the resource-poor countries. In this study, we report on our field and laboratory surveillance efforts in Nigeria. A total of 1,821 tissue samples, 8,638 tracheal swabs, 7,976 cloacal swabs and 7,328 avian sera were analysed over a period of two years, with 312 positive results.* We recovered 299 isolates of highly pathogenic avian influenza virus H5N1 mainly from the diagnostic samples of poultry kept in backyard, small scale and free range farms. This finding emphasised the role played by these farming systems in the dissemination of avian influenza in Nigeria and highlights the need for a continued surveillance in humans since human-animal interaction is a key feature in Africa. Furthermore, there is a need for the strengthening of border controls. Since October 2007, there has been no reported and confirmed outbreak of avian influenza in Nigeria.

Introduction

In late 1996, a farm in Guangdong, China was affected by infections with highly pathogenic avian influenza (HPAI) virus H5N1 [1,2]. Since the time of these reports, several countries in Asia (n=17), Europe (n=27), the Middle East (n=7) and Africa (n=11) have reported infection or re-infection of poultry flocks and/or wild and migratory birds [3].

In parts of the continents that reported infection, with the exception of Europe, it has been documented that the virus is becoming entrenched in the poultry populations and many clades and sub-clades are emerging [4]. Several hundred human infections (n=372) including 235 fatalities have similarly been confirmed [5], and most of these human infections have been linked to exposure to domestic poultry [6].

The expanding geography (infection of new locations), biology (acquisition of new biological properties) and ecology (adaptation to new host range) of H5 influenza viruses necessitated that every country should actively search for H5 avian influenza viruses within its territories. Nigeria, a country with an estimated human population of over 140 million, first reported infection in poultry in January 2006 [7], and in humans in January 2007 [5], and since that time, efforts to carry out active surveillance for the influenza viruses have been intensified by the national authority. Poultry production is a

key economic activity in Nigeria. It contributes significantly to the family income, especially in peri-urban and poor rural communities [8]. The effect of growing urbanisation the rural, peri-urban and urban poultry production and on human-animal interaction has previously been reported [9]. Backyard poultry production thrives in view of the level of poverty and the economic return associated with the venture. Free-range systems of poultry production are also widespread in various parts of the country [10].

Due to H5N1 avian influenza infection in Nigeria, millions of poultry have been destroyed and one human death has occurred. A recent serological survey in humans in those administrative regions in Nigeria that were most heavily affected by HPAI H5N1 showed that, despite the widespread infection in the poultry population, human infection is rare [11]. In this report, we describe our surveillance efforts in Nigeria and discuss the role of poultry and backyard flocks and their implications for humans vis-à-vis our laboratory findings.

Materials and methods

Poultry surveillance on farms and live bird markets

System 1 (October to December 2007). Based on available records, a stratified sampling with cluster sampling within each strata was adopted that included locations around previously infected farm premises and live bird markets as well as locations with suspected outbreaks and dense poultry populations. Each state of Nigeria was visited three times at intervals of two weeks, and samples were taken at two new locations during every visit. At each location, cloacal, tracheal and serum samples were taken from 29 birds, and six moribund, clinically ill or dead birds were purchased. All samples were transported in appropriate media and the cold chain was maintained throughout the activities.

System 2 (May to July 2008). The national active surveillance covered all 36 Nigerian states and the Federal Capital Territory (FCT), irrespective of whether or not HPAI H5N1 infections had been reported from the area, but was carried out in two parts: Part A of the targeted live bird market surveillance covered only the states with infections (25 states and FCT), while part B covered the 11 states without infections. This targeted surveillance programme is still ongoing.

System 3 (February 2006 to December 2007). While these activities were going on, additional routine diagnostic samples

(mostly tissue samples) were submitted to the National Veterinary Research Institute (NVRI) or collected in the field by the NVRI staff.

National surveillance programmes and team

In response to the outbreak of H5N1 influenza in the poultry population in 2006, the Nigerian government set up an inter-ministerial committee comprising health (Federal Ministry of Health), veterinary/agricultural (Federal Ministry of Agriculture and Rural Development) and information personnel (Federal Ministry of Information) to tackle the growing problem. Several routine surveillance efforts were jointly carried out at various times by the national teams in collaboration with representatives from the Food and Agricultural Organisation of the United Nations (FAO), the United States Centers for Disease Control and Prevention (US CDC), the World Organisation for Animal Health (OIE), the Istituto Zooprofilattico Sperimentale delle Venezie (IZSVE) and others. Teams were regularly dispatched to suspected farms nationwide to collect samples and identify infected birds, advise on compensations and carry out cullings.

Sample collection, virus isolation and serology from avian species

Following sample collection, post mortem examinations were conducted on birds acquired moribund, dead or freshly killed, and on tracheas, lungs, livers, spleens, brains, hearts, intestines as well as intestinal contents were collected in sterile containers.

Virus isolation was done in 9-11-day-old embryonated chicken eggs according to standard protocols [12]. The eggs were candled daily to determine viability and dead eggs were removed and kept at +4°C. All eggs were opened aseptically and the allantoic fluids (ALF) were spot-tested by haemagglutination test. The chorio-allantoic membranes (CAM) of positive eggs were tested by agar-gel immunodiffusion (AGID) to detect influenza A virus group antigen.

Haemagglutination-inhibition (HI) test was conducted to determine the virus subtype. All negative ALF were further passaged in a second set of embryonated chicken eggs. Any samples negative after the second passage were declared negative. As of May 2008, no isolates of influenza A virus have been obtained from the second passage**.

Serological assays including AGID test using the H5 antigen and HI test using standardized H5, H7 and H9 panels of antigens (OIE reference laboratory for Newcastle disease virus and avian influenza, Padova) were conducted on all sera submitted to the laboratory.

Molecular analysis

Viral RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR) were carried out. A cascade-type analysis was performed starting with the gene for the viral matrix protein (M). Every positive result was subjected to an RT-PCR for the haemagglutinin gene (HA) of subtype H5 and an additional RT-PCR for the N1 gene was done for all HA-positive cases. The following oligonucleotide primers were used: M forward: 5'-AGA TGA GTC TTC TAA CCG AGG TCG-3'; M reverse: 5'-TGC AAA AAC ATC TTC AAG TCT CTG-3'; H5 forward: 5'-CCT CCA GAR TAT GCM TAY AAA ATT GTC-3'; H5 reverse: 5'-TAC CAA CCG TCT ACC ATK CCY-3'.

Conventional RT-PCR has been shown to detect titre as low as 3 EID₅₀ (Fifty percent egg infectious dose). In addition, our results were confirmed by the OIE reference laboratory for avian influenza and Newcastle disease, Padova, Italy.

Human sero-epidemiological surveillance

Several locations (poultry farms, live bird markets) with suspected or confirmed HPAI H5N1 infections were visited (between 21 March and 3 April 2007) following the compilation of a list of affected areas by the Federal Ministry of Agriculture and Rural Development in Nigeria. Specifically, a total of 295 poultry workers (76% farm workers, 15% market workers, 5% poultry cullers and 4% veterinarians), from 83 farms and four live bird markets in Kano state, and 25 laboratory workers were included in the surveillance.

In addition, surveillance in humans had been carried out by Ortiz *et al.* between 21 March and 3 April 2006 [11]. In that study, human sera had been collected with the informed consent of participating individuals. In addition, serum samples had been collected from people potentially exposed to the HPAI H5N1 virus, including laboratory workers, veterinarians and culling staff that agreed to participate in the sero-survey. The blood samples had been transported on ice to the laboratory (Institute of Human Virology, Abuja). Sera had been prepared in the Human Virology Laboratory, Abuja, and split in two aliquots, one of which was kept for the Federal Ministry of Health while the other one was sent to the US CDC for H5N1 serologic testing. The human sera had been tested by microneutralisation assay and a modified horse red blood cell haemagglutination-inhibition (HRBC H-I) assay. For details see Ortiz *et al.* [11]*.

Results

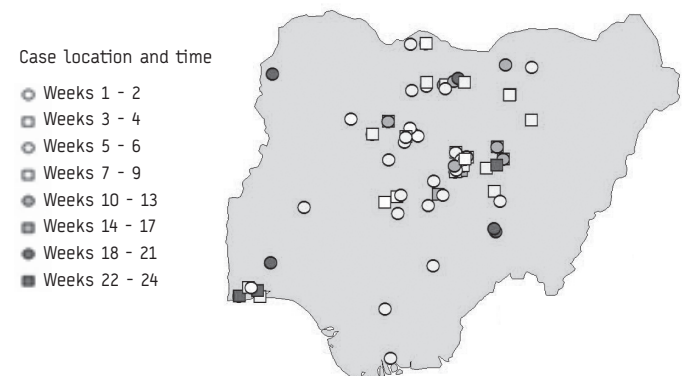
Poultry sero-surveillance

In the period between 2006 and 2007, farms located in 25 Nigerian states and the FCT reported poultry infections with HPAI H5N1 virus. The geographical distribution of the positive cases is shown in Figure 1.

Details of the results are shown in the tables below. During the two-year study period from January 2006 to December 2007, a total of 1,205 suspected routine diagnostic samples, 8,638 cloacal swabs, 7,976 tracheal swabs, 7,328 sera and 616 carcasses were received either from the field staff or directly from the farmers.

FIGURE 1

Temporal and geographical distribution of highly pathogenic avian influenza H5N1 poultry cases, Nigeria, January-June 2006 (n=113)



The surveillance in birds was carried out in the whole country with particular attention paid to the infected locations and live bird markets around them. Further 186 cases occurred between June and December 2007, bringing it to a total of 299 cases, but the overall geographical distribution did not change, with additional infections happening only in already affected locations.

The samples submitted as part of routine surveillance (system 3) yielded 300 positive results (Table 1).

Table 2 shows the results of the national surveillance using stratified a sampling procedure covering farms and live bird markets in the 36 Nigerian states and FCT (system 1). To date, all of these 10,961 samples have been negative.

For the targeted live bird market surveillance (system 2), results are available for part A covering only the 25 infected states and the FCT (Table 3). A total of 13,597 samples were analysed, of which 12 were found to be positive. The targeted live bird market surveillance for the 11 states without report of avian influenza infections (part B) is ongoing.

In the period from January 2006 to December 2007, 299 isolates of HPAI H5N1 were obtained and characterised. The haemagglutinin genes of 52 isolates have been sequenced and deposited in the GenBank and EMBL databases [13]. All of the positive isolates that were characterised belonged to clade 2.2. Efforts to genetically characterise more of the remaining isolates are currently underway.

TABLE 1
Avian diagnostic samples tested in Nigeria between 2006 and 2007

	Suspected total number	Positive samples
Diagnostic samples (tissues/swabs) tested in 2006	619	145
Diagnostic samples (tissues/swabs) tested in 2007	586	154 + 1*
Total	1,205	299 + 1*

Note: 52 isolates have been fully sequenced and are published [13]. A large majority (98%) of the isolates originated from farms. 1* represents a sample from Benin republic diagnosed in Nigeria.

TABLE 2
National active surveillance covering the 36 states and the Federal Capital Territory, Nigeria, October 2007 to July 2008 (n=10,961 samples)

Samples Collected	Number analysed	Number positive
Tracheal swabs	4,253	0
Cloacal swabs	3,608	0
Sera	3,100	0

TABLE 3
Targeted live bird market surveillance covering 25 states and the Federal Capital Territory, Nigeria, October-November 2007 (n=13,597 samples)

Samples Collected	Number analysed	Number positive
Tracheal swabs	4,385	3
Cloacal swabs	4,368	0
Sera	4,228	6
Carcasses and moribund birds	616	3

Tables 1-3 reveal a certain pattern in that H5N1 influenza virus isolates were obtained mainly from routinely submitted diagnostic samples and live bird markets. Following infection of farms, farmers promptly report outbreaks to the NVRI or other appropriate government agencies since this will ensure payment of compensation. However, we are aware that the level of education may affect reporting in certain circumstances and our systems may have inadvertently missed some outbreak situations. It is also very likely that viruses that escape detection at the farm level will get to the live bird market and can be detected there. These two locations (farms and live bird markets) are important in the epidemiology of avian influenza viruses in Africa.

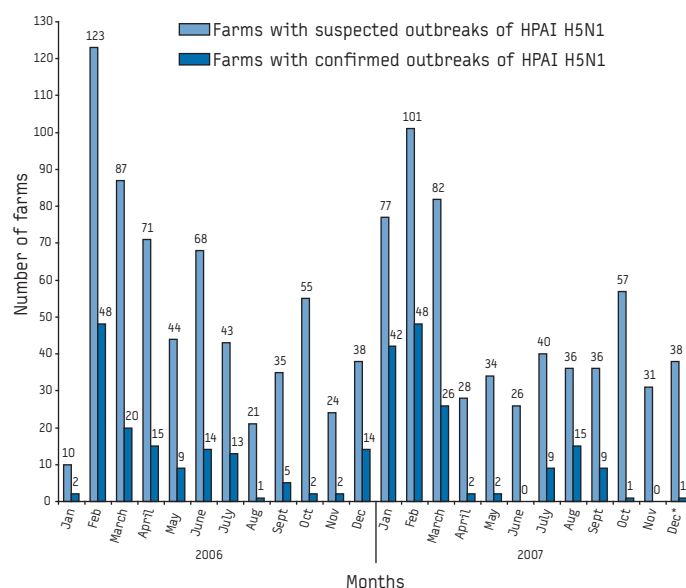
Figure 2 gives an overview on HPAI H5N1 outbreaks in the years 2006 and 2007 as determined by the routine diagnostic poultry surveillance.

The overall rate of confirmed outbreaks was 24.8%. The peaks of infection around January and February in both years may be linked to poultry movement which is usually on the increase around festive periods (December/January). The peaks in the June/July 2006 and July/August 2007 period similarly represent the times when seasonal guineafowl eggs are available. The same period is accompanied by sale of commercial poultry due to a surplus in the egg market caused by the cheaper guineafowl eggs.

Human sero-surveillance

As previously reported, none of the 320 human serum samples tested was positive for H5N1 avian influenza by micro-neutralisation assay or HRBC H-I test despite the degree of possible exposure to H5N1 influenza virus [11]*.

FIGURE 2
Suspected and confirmed outbreaks of HPAI H5N1 from routine diagnostic samples of avian species in Nigeria, 2006-2007 (n=1,205 reports and 299 confirmations)



2007 Dec*: The only isolate in that month originated from Benin Republic. HPAI: Highly pathogenic avian influenza.

Discussion

Before the first occurrence of avian influenza in Nigeria, active surveillance on wild fowl and migrating birds was conducted between September and November 2005 (results not shown) at the Nguru-Hadejia wetlands covering an area of about 4,125 km². Similar surveillance was done in the same period in the high risk agroecological/farming areas and live poultry markets, but failed to detect H5 or H7 avian influenza virus.

However, after the first avian influenza outbreak in Nigeria in January 2006, surveillance efforts in the period between January, 2006 and December, 2007 yielded a total of 299 Nigerian isolates of HPAI H5N1. Mutations at antigenic sites were identified in the haemagglutinin genes of the viruses, the significance of which need to be confirmed by further analyses. The implications of these mutations for human and animal health is yet unknown [13]. Although the H5N1 virus has not yet adapted to effectively infect humans, there remains a potential pandemic threat in view of continuous infections on farms in the West African sub-region. Furthermore, there is a need to carry out routine surveillance for other influenza viruses in human and animals, since a recent report using animal models indicated that the H9N2 influenza virus showed increasing pandemic potential [14].

We are aware that our surveillance systems are subject to certain limitations. Firstly, the systems were limited and not all locations within each state were considered. In addition, some bias may be caused by the fact that the surveillance of birds may not be possible in difficult terrains. However, we made every effort to give priority to locations that serve as points of aggregation of poultry products from many locations.

We may have underdetected some cases in view of the availability of more robust and sensitive analytic systems like real time RT-PCR, and are currently making an effort to put in place such an analytic system. It was also difficult to get paired serum samples in most locations since farmers were free to dispose of their birds without regards to the on-going surveillance.

Despite these limitations, we think that this nationwide effort is critical and important since Sub Saharan Africa faces many challenges of controlling and eradicating H5N1 in poultry and implementing a good surveillance system for H5N1 in humans.

The human sero-epidemiological survey reported by JR Ortiz *et al.* did not detect any human H5N1 infections in Nigeria [11]*. This result is similar to the data recorded in previous studies in Cambodia (0/351) and Guangdong, China (1/110) [15,16]. This probably confirms that the virus has not yet adapted to effectively infect humans.

Although the human serosurveillance was negative, human H5N1 infections in Nigeria cannot be excluded. It is common practice in the northern part of the country, for reasons of culture, religion and poverty, to bury a deceased person within 24 hours of death, sometimes without ascertaining the cause of death through post mortem and detailed laboratory examinations. The only human case in Nigeria, which was officially reported by the World Health Organization on 3 February 2007, was diagnosed following a thorough investigation of a fever complicated by respiratory distress which finally led to death. It is important to ensure in the future that at least diagnostic specimens are collected before burial for proper retrospective analysis. Since it is beyond the mandate of NVRI to do a nation-wide serosurveillance in humans, the Nigerian

Federal Ministry of Health, human medical practitioners, virologists and immunologists are encouraged to carry out a similar study in humans in Nigeria and parts of the West African sub-region.

Globalisation can affect animal and human health and change the disease ecology especially in those countries that presently claim to be free from HPAI infection in humans and animals [17], and risk assessment studies have shown that the European Union and parts of North America are at high risk of infection with animal diseases, in particular those originating from Africa [18-21]. These countries will need to strengthen their borders with respect to animal disease controls.

To date, the majority of the HPAI H5N1 cases in Europe has been introduced through wild birds. The source of contamination as well as the movement pattern of these wild and migratory birds needs to be studied more critically in order to exclude cross-continent infection of a potentially pandemic influenza virus.

Since October 2007, there has been no confirmed outbreak in Nigeria despite the on-going intensive surveillance. This situation has helped to stabilise the Nigerian poultry industry and has had a positive psychological effect on consumers. However, the continued absence of HPAI H5N1 will depend on sustained surveillance of poultry farms and live-bird markets, changed agricultural practices and a heightened biosecurity system entrenched in the farming system in Nigeria. Cross-continent collaborative research is encouraged and a network of funding systems, especially from the rich countries, to support research and diagnosis in developing economies like Nigeria will be greatly valued.

** Note added in proof: Since the time of submission of this report, the FAO laboratory has recently (June and July, 2008) isolated and molecularly characterised new HPAI virus isolates obtained from live bird markets and from outbreaks in farms in a total of four Nigerian states. While the viruses from two states, Kano and Katsina, (isolated from farms) belonged to the old clade (2.2) circulating in Nigeria, the isolates from two other states, Gombe and Kebbi, belonged to a new sublineage of clade 2.2, EMA3, that is novel to the African continent. This sublineage was previously circulating in Europe (Italy), Asia (Afghanistan) and the Middle East (Iran) in 2006.

* Erratum: The following amendments were made to correct the fact that supporting data on sero-surveillance in humans had mistakenly not clearly been labelled as cited from a previous publication: The sentence "Limited human sero-surveillance involving 320 individuals was also carried out but yielded no positive results" was removed from the abstract. The paragraph "Surveillance in humans was carried out between 21 March and 3 April 2006 [11]. Human sera were collected with the informed consent of participating individuals. In addition, serum samples were collected from people potentially exposed to the HPAI H5N1 virus including laboratory workers, veterinarians and culling staff that agreed to participate in the sero-survey. The blood samples were transported on ice to the laboratory (Institute of Human Virology, Abuja). Sera were prepared in the Human Virology Laboratory, Abuja, and split in two aliquots, one of which was kept for the Federal Ministry of Health while the other one was sent to the US CDC for H5N1 serologic testing. The human sera were tested by microneutralisation assay and a modified horse red blood cell haemagglutination-inhibition (HRBC H-I) assay. Details of the tests have been reported comprehensively in another paper [11]." was changed to "In addition, surveillance in humans had been carried out by Ortiz *et al.* between 21 March and 3 April 2006 [11]. In that study, human sera had been collected with the informed consent of participating individuals. In addition, serum samples had been collected from people potentially exposed to the HPAI H5N1 virus, including laboratory workers, veterinarians and culling staff that agreed to participate in the sero-survey. The blood samples had been transported on ice to the laboratory (Institute of Human Virology, Abuja). Sera had been prepared in the Human Virology Laboratory, Abuja, and split in two aliquots, one of which was kept for the Federal Ministry of Health while the other one was sent to the US CDC for H5N1 serologic testing. The human sera had been tested by microneutralisation assay

and a modified horse red blood cell haemagglutination-inhibition (HRBC H-I) assay. For details see Ortiz et al. [11].” The sentence “None of the 320 human serum samples tested was positive for H5N1 avian influenza by micro-neutralisation assay or HRBC H-I test ...” was changed to “As previously reported, none of the 320 human serum samples tested was positive for H5N1 avian influenza by micro-neutralisation assay or HRBC H-I test ... [11].” The sentence “The human sero-epidemiological survey reported in this study did not detect any human H5N1 infections in Nigeria.” was changed to “The human sero-epidemiological survey reported by JR Ortiz et al. did not detect any human H5N1 infections in Nigeria [11].”

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