Influenza virus A (H1 and H3) and B co-circulation among patient presenting with acute respiratory tract infection in Ibadan, Nigeria

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Abstract

Background: Influenza is an acute respiratory disease that continues to cause global epidemics and pandemics in human with significant mortality and morbidity.

Objectives: This study was designed to identify the circulating influenza virus in Ibadan, Nigeria during the 2006/2007 season.

Methods: Throat swab samples were collected from patients presenting with acute respiratory tract infection at the Out-Patient Departments of major hospitals in Ibadan over a period of seven months from November 2006 to May 2007. Isolation of influenza virus was performed using Madin-Darby Canine Kidney cell line and 10 days old chicken embryonated egg. Isolates was identified by haemagglutination and haemagglutination-inhibition assays using selected CDC Influenza reference antisera (A, B, subtype H1 and H3).

Results: Out of 128 patients tested, 21(16.4%) yielded positive for virus isolation. Identification of the isolates showed that 19(14.8%) were positive for influenza virus out of which 11(8.6%) and 8(6.2%) were influenza A and B viruses respectively. Influenza A virus 6(4.7%) were subtype H1; 4(3.1%) were co-subtype H1 and H3; and 1(0.8%) was not inhibited by subtype H1 and H3.

Conclusion: The circulation of influenza virus A and B in this study is important to contributing knowledge and data to influenza epidemiology and surveillance in Nigeria.

Keywords: Influenza A, haemagglutination, isolation, Nigeria.

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Introduction

Influenza is an acute respiratory disease that continues to cause global epidemics and pandemics in humans with significant mortality and morbidity reported and documented every season[1]. Three distinct immunologic types of influenza virus A, B and C have been classified[2]. The three major pandemics of the twentieth century were caused by influenza virus type A, a member in the family Orthomyxoviridae. Influenza A viruses are classified into 18 haemagglutinin (H1-H18) and 11 neuraminidase (N1-N11) transmembrane proteins based on the antigenicity of these glycoproteins[3]. Epidemics are usually caused by influenza virus type A or B. Type A virus can infect a variety of different host species including man, birds, pigs, horses and other animals. Influenza type B and C viruses only infect humans; however, influenza type C virus has been isolated in pigs[4] and dogs[5,6].

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The pandemics of influenza are typically due to the emergence of 'subtypes' of influenza A viruses as influenza B and C viruses have not been known to cause pandemic in the past[7]. When a significant "antigenic" shift in at least one of the influenza A virus surface proteins haemagglutinin and neuraminidase occurs spontaneously, often there is no immunity to this new virus. This can lead to a pandemic if the virus also achieved effective human to human transmission and replication causing serious illness. This can occur as it happened with Spanish flu caused by influenza virus of the A/H1N1 subtype in 1918[8,9]. Evidence has shown that multiple lineage of the same virus subtype can co-circulate, persist and re-assort in epidemiologically significant ways[10]. The tendency of influenza virus to undergo frequent and permanent antigenic changes necessitates constant monitoring of the global situation and annual adjustments in the composition of influenza vaccines[11]. The World Health Organization (WHO) epidemiological models indicate that a pandemic would have the greatest impact on the poorest countries of Asia and Africa as a result of limited surveillance[11].

In 2001, the WHO African Region's Integrated Disease Surveillance and Response strategy was implemented in Nigeria but the surveillance program captures inadequate data on influenza[12]. The public health programs in Nigeria have focused on avian influenza virus surveillance in animals as a result of episodes of zoonotic transmission of avian influenza viruses in wild birds and poultry[13,14]. Human living in close proximity to domestic poultry and pigs increases the risk of influenza to human health. Therefore, the Nigerian Federal Ministry of Health extended influenza surveillance in 2008 in order to acquire epidemiologic and laboratory data on the influenza disease among humans and the circulating influenza virus types and subtypes[15]. Previous publications have reported serologic evidence of influenza viruses circulating among humans in Nigeria[13,15-21]. There is however, urgent need for active surveillance and continuous monitoring of circulation of influenza virus in humans for influenza pandemic preparedness and control in Nigeria and sub-Sahara African countries.

Early warning of the emergence of a new strain requires active surveillance in which the general practitioners can play an important role. When surveillance is kept upon a community by records of illness and repeated virological tests, it is possible to detect sporadic instances of influenza virus infection. The detection and isolation of endemic and circulating influenza viruses will aid in the formulation of effective vaccines and the development of suitable method of vaccination. Therefore, this study isolated and determined the circulating type and strains of influenza virus from patients with acute respiratory tract infections (RTI) using haemagglutination (HA) and haemagglutination-inhibition (HI) assays for 2006/2007 season in Ibadan, Nigeria.

Materials and method

Study population

The study was discussed and approved by the local Ethics Committee. Throat swab samples were collected at the General Out-Patient Departments of three regional hospitals in Ibadan, the South-Western part of Nigeria (Figure 1) from November 2006 to May 2007 (dry to early onset of raining season). All laboratory investigations were carried out at the Department of Virology, College of Medicine Ibadan. Acute RTI was defined as clinical presentation of sudden onset of fever (temperature ≥38.0°C), cough/sore throat, fatigue and sputum production, in any adult or child seeking care. Patients with acute RTI irrespective of their age were eligible for sampling as long as the onset of symptoms was within three days. Information on demographic characteristics, medical history and clinical signs such as muscle aches, chills, malaise, vomiting, abdominal pains, nasal stiffness, runny nose, headache, diarrhoea and neurological manifestations were recorded during patient's enrolment.

Figure 1: Location of the influenza study (indicated in black dot) in Ibadan, South-Western part of Nigeria

Informed consent was obtained from all patient or patient's parents in the case of children prior to sample collection. Throat swabs were carefully collected from 128 patients by trained clinicians using polyester fiber-tipped applicator to rub the posterior of the tonsils, the soft palate and posterior wall of the pharynx. The swabs were then carefully removed; the cotton tip of swab stick was broken off into a labelled screw-cap vial containing virus transport medium (Dulbecco's modified eagle medium, 2% Bovine serum albumin, 2% of HEPES buffer and 100 μg/ml of penicillin/streptomycin). Samples were quickly transferred in a box containing ice packs to maintain cold-chain during the course of transportation to the laboratory where they were inoculated immediately or stored under mechanical refrigeration at -80°C for longer than 24 h before analysis. However, prior to inoculation, the throat swab samples in transport medium were treated with 100 μl of antibiotics made of penicillin and streptomycin in a laminar flow hood. This was left in a temperature of 4°C for 1 hour. The mixture was then filtered by centrifugation in the cold at 1500 rpm to ensure separation of debris from the fluid. The treated samples were then used for inoculation.

Virus isolation

Detection of cytopathic effect (CPE) in cell line

Aliquots (100 μl) of the throat swab samples were inoculated into the Madin-Darby Canine Kidney cell line (MDCK; WHO/CDC, USA) containing confluent cells previously seeded in tubes or microplates. Each sample was inoculated in duplicate and two tubes left not inoculated to serve as control. Inoculated culture tubes were incubated at 37°C and daily observation for CPE under inverted x10 microscope for 7-8 days was recorded accordingly. Cultures showing CPE were harvested when they had progressed to about 75% to 100% and subsequently passaged. Cultures with no visible CPE at the end of day 7 were also retrieved for blind passage while samples not showing CPE after second passage were regarded as negative. Cultures with CPE after second passage were further passed into MDCK in tissue culture flasks to increase the virus titer (50%). The CPE observed include cell granulation, swelling, cell fragmentation and dislodgement of the cell monolayer.

Inoculation and harvesting of embryonated egg

A small hole was drilled above the air space in the shell of 10 days old chicken embryonated eggs obtained from local hatchery. A 23-gauge needle attached to 1 ml syringe was inserted into the amniotic sac and 100 μl of the throat swab inoculum was injected into the amniotic cavity. An additional 200 μl inoculum was injected into the allantoic cavity. Two eggs were inoculated per sample. Two eggs were uninoculated to serve as control. The

hole in the shell was thoroughly sealed with wax, labelled appropriately with date and incubated at 37°C for 3 days. Eggs were monitored daily by candling to determine the viability of the eggs. Following incubation, eggs were chilled at 4°C for 4-24 hours to minimize bleeding during harvesting. The allantoic fluid was then harvested with a Pasteur pipette over the air sac and the amniotic fluid, with a short 23-guage needle attached to a 1 ml syringe. Samples with no haemagglutinating activity were harvested to passage into another set of eggs. Two passages were performed before considering a sample negative for influenza virus isolation.

Haemagglutination and haemagglutination-Inhibition assays

Haemagglutination assay was performed on samples serially diluted in 2-fold steps in a microtiter plate using 0.5% washed chicken red blood cell (RBC) as indicator system including the controls (containing phosphate buffer saline and RBC). The plates were slightly agitated and then incubated undisturbed at room temperature for 30 minutes after which the plates were observed for agglutinatination. Positive haemagglutinating viruses were further tested quantitatively by calculating the 1HA and 4HA unit titers used to prepare the working virus dilution needed for haemagglutination-Inhibition (HI) assay. Those with high 4HA (>22) titers were harvested and stored in aliquots at -80°C. The haemagglutination inhibition of the unknown virus was identified using ready-to-use reference antisera and antigen: FR-1217- and FR-1218- mouse monoclonal antibody influenza types A and B (pool) respectively; FR-54- and FR-572- monoclonal antibody influenza types A/H1 and A/H3 respectively (WHO-Collaborating Centre for Influenza/CDC, Atlanta Georgia 30333, USA) provided in the WHO/CDC Influenza surveillance kit. Positive Influenza A and B viruses were identified according to WHO protocol. As different reference antisera were used, the ability of the virus to bind to specific antiserum shows the type of influenza virus either type A or B. The HI test is considered valid if the positive reference antigen and its homologous antiserum demonstrate the expected HI titer and the back titration of each antigen (unknown and positive control) is 4 HA units/25 μl.

Results

Fever, sputum production, cough, malaise, fatigue, runny nose and headache were common clinical manifestations observed in positive patients enrolled for the study. All patients with HA antibody to influenza virus tested by HI assay as described above presented with fever 19(100%). In this study, out of 62 male and 66 female patients tested, 9(14.5%) and 10(15.2%) were positive for influenza virus infection respectively. Within the age group of 0–5 years old, 14(12.8%) patients were tested positive for influenza virus while in the age groups 16-20 and 21-25 years old; 1(100%) and 2(40%) patients were tested positive for influenza virus respectively (Table 1).

In November 2006, out of 37 patients with acute respiratory infection tested for influenza virus infection, 7(18.9%) were positive for influenza virus infection (Table 2). The number of positive patients diminished over the month of collection until May 2007, when it began to rise again.

Table 1: Age distribution of tested and positive patients with influenza virus infection

Age group (years)	No of samples tested	No (%) of positive
0 - 5	109	14(12.8)
6 -10	4	2(50)
11 – 15	2	0
16 - 20	1	1(100)
21 - 25	5	2(40)
26 – 30	4	0
≥ 31	3	0
Total	**128**	**19(14.8)**

Table 2: Monthly distribution of tested and positive patients with influenza virus infection

Month	No of samples tested	No (%) of positive
November 2006	37	7(18.9)
December 2006	35	4(11.4)
January 2007	20	4(20)
February 2007	14	3(21.4)
March 2007	10	0
April 2007	5	0
May 2007	7	1(14.3)
Total	**128**	**19(14.8)**

In this study, out of 128 clinical samples collected from patients with respiratory infection, 21(16.4%) were positive for virus isolation in MDCK cell line and/or embryonated egg cultures (Table S1). Out of the 21 positive samples, 7(5.5%) and 18(14.1%) samples yielded positive in MDCK and embryonated egg cultures respectively while 4(3.1%) samples were positive in both culturing systems. Identification of the isolates with CDC reference antisera (Influenza type A, B, subtype H1 and H3) showed that 19(14.8%) were positive for influenza virus out of which 11(8.6%) was identified as influenza A virus and 8(6.2%) as influenza B virus. Further analysis showed that for influenza A virus of which 6(4.75%) were subtype H1 and 4(3.1%) were co-subtype H1 and H3, 1(0.8%) was not inhibited by subtype H1 and H3 as identified by HI. Out of the four samples positive in both culturing systems,

two were influenza type A/H1 and H3; one was influenza type B; and one was influenza type A (not subtype). Two of the isolates that haemagglutinated chicken RBC could not be identified with the available antisera A and B (Table S1). These isolates were sent to WHO collaborating Centre in Atlanta for further molecular investigation.

Table S1: Virus isolation and identification of positive samples

Sample No	Virus Antisera	Date of Isolation	Positive MDCK	Embryonated Egg	Clinical signs
NIG-FLU-0129	A/H1/H3	November 2006	Yes	Yes	Fever, sputum production, cough, malaise, fatigue, runny nose, headache.
NIG-FLU-0130	A/H1	November 2006	No	Yes	Fever, sputum production, cough, malaise, fatigue, runny nose, headache, sore throat.
NIG-FLU-0131	B	November 2006	No	Yes	Fever, cough, runny nose, headache, sore throat.
NIG-FLU-0132	B	November 2006	No	Yes	Fever, sputum production, cough, malaise, fatigue, sore throat.
NIG-FLU-0137	A/H1	November 2006	No	Yes	Fever, sputum production, cough, malaise, fatigue, runny nose, headache.
NIG-FLU-0140	A/H1	November 2006	No	Yes	Fever, sputum production, cough, malaise, fatigue, runny nose, headache.
NIG-FLU-0141	A/H1	November 2006	No	Yes	Fever, sputum production, cough, malaise, fatigue, runny nose, headache, sore throat.
NIG-FLU-0149	B	December 2006	No	Yes	Fever, sputum production, cough, malaise, fatigue
NIG-FLU-0172	B	December 2006	Yes	No	Fever, sputum production, cough, malaise, fatigue
NIG-FLU-0179	A/H1/H3	December 2006	Yes	No	Fever, sputum production, cough, runny nose, headache.
NIG-FLU-0180	*A	December 2006	Yes	Yes	Fever, sputum production, cough, malaise, fatigue, runny nose, headache, sore throat.
NIG-FLU-0187	A/H1/H3	January 2007	Yes	Yes	Fever, sputum production, cough, malaise, fatigue, runny nose, headache.
NIG-FLU-0189	B	January 2007	Yes	Yes	Fever, sputum production, runny nose, headache.
NIG-FLU-0191	A/H1/H3	January 2007	Yes	No	Fever, sputum production, cough, malaise, fatigue, runny nose, headache.
NIG-FLU-0216	A/H1	January 2007	No	Yes	Fever, sputum production, cough, malaise, runny nose, headache.
NIG-FLU-0219	A/H1	February 2007	No	Yes	Fever, sputum production, cough, malaise, fatigue, runny nose, headache, sore throat.
NIG-FLU-0220	B	February 2007	No	Yes	Fever, sputum production, cough, runny nose,
NIG-FLU-0222	B	February 2007	No	Yes	Fever, sputum production, malaise, fatigue, headache.
NIG-FLU-0231	-	May 2007	No	Yes	Fever, sputum production, cough, sore throat
NIG-FLU-0234	B	May 2007	No	Yes	Fever, sputum production, cough, malaise, fatigue
NIG-FLU-0256	-	May 2007	No	Yes	Fever, sputum production,

* Unable to subtype
- Unable to type

Discussion

In this study, influenza A virus was the type most frequently isolated and identified within the study population in 2006/2007 season. Previous reports have shown that Influenza A is predominantly in circulation in Nigeria[15-21] which corroborates with findings from other sub-Saharan African countries[22,23]. Influenza A virus was the most common circulating influenza virus type identified within Nigeria in 2009 pandemic[20,21]. Information on worldwide influenza surveillance data accessed from the WHO database (FluNet) was used to compare the influenza type that predominates in Nigeria with those in other sub-Saharan African countries for 2006/2007 season. Regrettably, most of these countries including Nigeria had no data available for 2006/2007. In 2006, however, influenza virus A/H1, A/H3 and A (not subtyped) predominated in Senegal, Cameroun and South Africa respectively. Contrarily, influenza B virus predominated in Kenya. In 2007, influenza virus type A (not subtyped) predominated in Kenya and South Africa; type A/H3 predominated in Cameroun; and type B predominated in Senegal[24]. Influenza surveillance in African countries is very limited, the seasonality and epidemiology of the influenza viruses need to be fully understood though. Importantly, pre- and post-pandemic data extending over years is required to understand why influenza virus B is less detected compared to type A influenza viruses, most particularly, in Nigeria. We recognized that the results of this study are not a representative of the entire population of Nigeria which may be a possible shortcoming. Nevertheless, to the best of our knowledge, it is the first study to provide insight to the type of human influenza virus circulating among patients presenting with respiratory infection in Nigeria in 2006/2007. In a study that was carried out at Olympic Village Polyclinic during the 2002 Winter Olympiad in Salt Lake City, USA in February and March 2002, 188 patients were screened for influenza and influenza A was detected in 28(15%) and influenza B in 8(4%) patients[25], showing predominance of influenza A. According to[26] Morbidity Mortality Weekly Report shows that influenza B is less common in circulation.

In this study, it was demonstrated that in patients positive to influenza virus infection, fever (100%) was frequent and consistent which corroborate the report by[27],[28] Centre for Disease Control reported that influenza virus infection is usually abrupt with symptoms of malaise, extreme fatigue, headache, cough rhinitis and sore throat. This is similar to our findings as the following symptoms were common; sputum production 18(94.7%). cough 16(84.2%), malaise 14(73.7%), fatigue 14(73.7%), headache 14(73.7%) and runny nose 14(73.7%). However, the number of positive patients with sore throat 6(31.6%) was rather low. This was because most of the patients sampled were children, within the age-group of 0–5 years and they were unable to describe the sensation of sore throat not even the parents. This at the same time was not obvious to the clinicians and some researchers.

Influenza virus is notoriously known for its unique ability to cause recurrent epidemics and pandemics during which acute febrile respiratory illness occurs explosively in all age-groups. In this study, a significant higher number of samples were collected from children in age group 0–5 years. Children aged less than 2 years were among the groups that are at risk of serious illness and death from influenza[17]. Although distribution of influenza virus type based on age group is not shown in our data, out of all 19 patients tested positive to influenza; a high number 14(12.8%) were detected in children of age group 0–5 years. It has been reported that children (5–17 years old) were most frequently infected with influenza virus20. This shows a high risk of susceptibility of children to the infection. This can be as a result of immune immaturity in neonate. More so, young children can shed copious number of virus in droplets by sneezing and coughing for longer days and can be infectious for over ten days[29]. Parent tends to show immediate attention by bringing their children to hospital immediately they show signs of illness. In our study, it was observed that adults usually regard flu-like symptoms as common cold, therefore not taking it serious to consult physicians until the condition becomes severe. At this period, they are often unsuitable for throat swab collection for virus isolation.

Genetic re-assortment can occur between two different subtypes of influenza A viruses resulting into pandemic[30]. Although, in this study, co-circulation of influenza A subtypes H1 and H3 was observed, it would be inappropriate to draw conclusion about the possibility of pandemic by influenza A virus subtype H1/H3 or emergence of a new subtype of influenza A virus on the basis of our

data. Multiple lineages of the same virus subtypes can co-circulate persistently and re-assort in an epidemiologically significant ways[10]. At present, H1 and H3 subtypes of influenza A viruses are the predominant type causing epidemic infections in the human population[29,31]. In our study, it is plausible that the two isolates uninhibited by antisera (A and B) may be influenza C or another haemaglutinating virus such as measles or mumps as they showed no CPE on MDCK. A limitation to this study, however, is the need for molecular investigation in order to identify unknown isolates, confirm the possibility of dual infection (H1 and H3), re-assortment and N subtypes.

Nigeria lies within the tropical zone of West Africa region. There are basically two distinct seasons in Nigeria namely the rainy/wet (April–October) and dry/harmattan (November–March) seasons influenced by maritime tropical air from the Atlantic Ocean and continental tropical air of the Sahara Desert respectively. Rainfall is the key climatic variable; there is a marked alternation of the two seasons in most areas though[32]. The seasonality of influenza viruses in the tropics has been associated with the rainy season[33,34,35]. In Nigeria, however, studies have suggested that influenza virus infection is more associated with the dry (harmattan) period[15,36]. In this study, the outbreak of influenza virus infection coincided with the onset of harmattan season (November to February). At the cessation of harmattan precisely March and April, patients tested were negative to influenza. This suggests that influenza virus spreads and thrives well in harmattan, giving a favourable condition forits transmission. This may explain why the number of positive patients was high between November 2006 and January 2007 (Table 2).

Conclusion

This study established the circulation of influenza A (H1 and H3) and B viruses within the study population in Ibadan, Nigeria. The rise of influenza B virus co-circulation may eventually displace and replace the dominant influenza A virus in Nigeria. Influenza virus contributes to the occurrence of mild cases of respiratory infection most especially in children. Although most cases of RTI were not positive for influenza, other respiratory diseases should be considered in influenza surveillance. The fact that mortality impact of influenza epidemics and pandemics is devastating; this study affirmed the importance and need for influenza surveillance within sub-Saharan Africa. Data from this study strengthens influenza surveillance system which can be integrated into the public health programs in Nigeria and sub-Sahara African countries. Regular and active continuous monitoring and surveillance by isolation, characterization and molecular sequencing through laboratory investigation is thus necessary to further enhance diagnostic and epidemiological data on influenza in Nigeria.

Supplementary material

Supplement data associated with this article is provided as (Supplemental) Table S1.

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Conflict of interest

The authors of this paper declare no conflict of interest.

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