

Serological evidence of equine influenza virus in horse stables in Kaduna, Nigeria

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Equine influenza virus (EIV) is a major cause of acute respiratory diseases in horses in most parts of the world that results in severe economic losses. Information on the epidemiology of EIV in tropical Africa is scanty. An enzyme-linked immunosorbent assay (ELISA) was used to detect the presence of influenza A virus nucleoprotein (NP) in 284 horse sera in Kaduna State, Northern Nigeria. The ELISA-positive sera were further examined for hemagglutination inhibition (HI) antibodies to two strains each of H3N8 and H7N3 subtypes of influenza A virus. The results showed that antibodies against influenza A virus nucleoprotein were detected in 60.9% (173 of 284) of horses examined by NP-ELISA. Equine H3 and H7 subtypes were detected in 60% (21 of 35) and 20% (7 of 35) of horse sera respectively across the stables. Adequate quarantine of all imported horses, a national equine influenza surveillance plan and an appropriate EIV control program in Nigeria are recommended to safeguard the large horse population.

Key words: equine, influenza virus, Nigeria, stables

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Equine influenza is a highly contagious, infectious upper respiratory disease of horses characterized by anorexia, pyrexia, dyspnea, dry cough and nasal discharges [19, 20, 41]. It is caused by the equine influenza virus (EIV), a respiratory pathogen of horses and other equidae belonging to the *Orthomyxoviridae* family (genus *Influenza A*).

Two EIV subtypes H7N7 (equine-1) and H3N8 (equine-2) are recognized to have been established in horses, co-circulating for nearly two decades [39]. The H7N7 subtype which was first isolated in horses in 1956 [35] has not been in circulation since 1978 [38], however, serological evidence of this virus subtype has been reported in India in the recent past [34]. The H3N8 (avian-origin influenza) which was first isolated in 1963 [41] has continued to spread panzootically among horses [9, 24]. To date, H3N8 subtype have been the cause of all outbreaks of EI since 1979 [8]. Evidence of the H3N8 subtype in dogs [7, 12] and pigs [37] has been

documented and H3N8 experimental infection has also been demonstrated in cats [33].

Pneumonia usually occurs in infected animals following secondary bacterial infection [30]. The disease is less fatal in the absence of pneumonia; however, an outbreak may cause suboptimal performance and devastating economic losses in the equine industry [8, 29, 42]. It is transmitted through direct contact and inhalation, with an incubation period of 2–5 days and an extremely high mortality of >90% [31, 43].

EI is endemic in Europe and North America and major outbreaks have been recorded in the recent past in Asia, that is, in China, Hong Kong, Japan, India and Mongolia [10, 29, 40, 44, 45]; in Africa, that is, in Nigeria, South Africa and Algeria [2, 11, 17]; in Australia [32] and in South America [25] affecting tens of thousands of horses. Recently, an outbreak of EI was confirmed in several horses between the ages of 1 to 7 years at a championship horse show in Oregon, U.S.A. [36]. To date, New Zealand and Iceland are reported to be free of EIV [25]. Information on the epidemiology of EIV in tropical Africa is scanty. In Nigeria, the first known outbreak of EIV occurred in 1991, among horses from different parts of the country congregated at the Ibadan polo tournament in the south western region [2]. From available records, some of the horses stabled in

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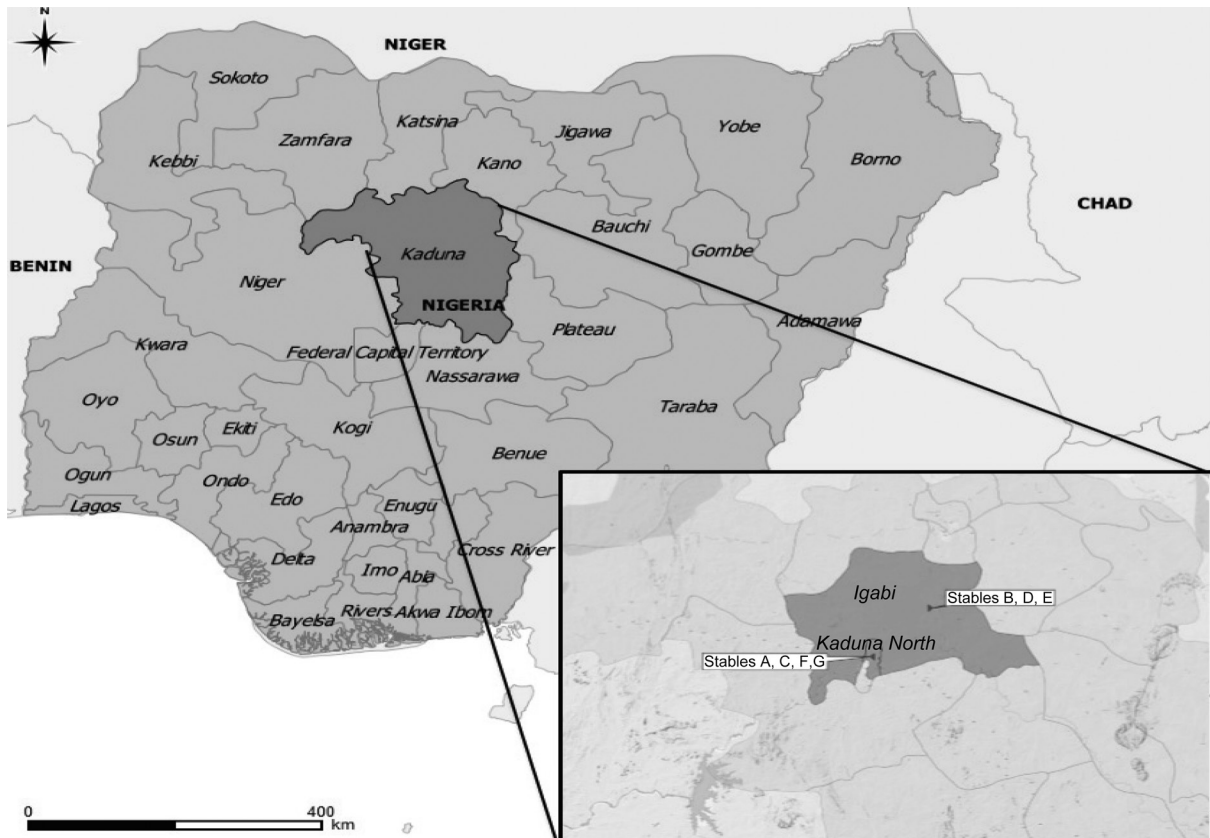


Fig. 1. Map of Nigeria showing study area and stable locations.

temporary shelters during the tournament were said to have been recently imported from Argentina and the U.K. This outbreak was caused by the H3N8 subtype. Although there had not been any reported EIV outbreak in Nigeria since the first outbreak, serological evidence of EIV subtypes H3 in horses [3, 27] and EIV subtype H7 in horses, pigs, chickens and humans [1, 25, 26] have been documented.

Natural human infection with equine influenza is rare, but experimental infection of human volunteers with equine influenza virus has been demonstrated [14]. A study has also shown an antigenic relationship between the equine and the Hong Kong human variants of the influenza subtype H2 virus [15]. Despite the occasional identification of seropositive individuals with occupational exposure there is currently little evidence of human infection with equine influenza [5, 18].

The emergence in 2009 of pandemic influenza from swine and its continuous evolution at the human-animal interface also underscore the need for sustained surveillance and monitoring of influenza virus in various susceptible species [21]. Only partial serological data are available for EIV in Nigeria, and serological testing has usually only been within specific areas in the south western Nigeria particu-

larly during polo tournaments. Moreover, there is bias due to the fact that samples obtained usually comes from horses within polo club stables. In this study, we carried out a cross-sectional serological survey of equine influenza in some horse stables within government security institutions, private institutions (polo clubs) and a traditional institution in Kaduna metropolis, north western Nigeria.

Materials and Methods

Description of study area and stable management

Kaduna state is located in the north western region of Nigeria, in the Northern Guinea Savannah zone (Fig. 1). Horses in seven different stables (designated A–G) belonging to government security institutions (n=2), private organizations (n=4) and a traditional institution (n=1) located within Kaduna metropolis, Nigeria were used for the study in January 2013. These stables were under intensive-semi-intensive management accommodating approximately 30–200 horses each. The horses were kept primarily for special ceremonial activities, trainings, crowd control, racing competition and polo. Routine veterinary care was provided for horses at the stables except stable

G, and no preventive vaccinations were routinely applied against EI or any animal influenza in Nigeria.

Sample collection

Using the principles of convenience sampling, about 50% of the total number of horses in each stable were selected for blood sampling. A total of 284 horses of all ages (2–22 years), different breeds and of both sexes representing these stables were sampled under proper restraint. Approximately 10 ml of blood was collected from each horse into properly labelled Vacutainer tubes and transported to the virology laboratory in National Veterinary Research Institute, Vom, Nigeria. The blood samples were allowed to clot, and then centrifuged in the laboratory at 2,000 rpm for 5 min. Sera were separated into properly labelled cryovials, and stored at -20°C until tested.

Test procedure: Enzyme-linked immunosorbent assay (ELISA)

An influenza A virus antibody ELISA test kit (IDEXX, Montpellier, France) was used according to the manufacturer's instructions for the measurement of the relative level of group-specific antibodies to influenza A nucleoprotein in the horse serum tested. Briefly, the test plate was pre-coated with influenza A viral nucleoprotein antigen. Upon incubation of the diluted samples (1/10), influenza A-specific antibodies form a complex with the coated nucleoprotein antigen. After washing away unbound material, an anti-influenza A nucleoprotein monoclonal antibody enzyme conjugate was added to the wells. If the sample did not contain influenza A nucleoprotein antibodies, the conjugate bound to the influenza A antigen on the plate. If antibodies to influenza A virus were present in the sample, the anti-influenza A nucleoprotein conjugate was blocked from binding to the antigen. Unbound materials were washed away and a colorimetric reaction was developed with the addition of an enzyme substrate. Optical density reading was performed with a spectrophotometer and a 650 nm filter. Results were evaluated based on the mean of sample to negative control mean (S/N) ratio. Samples with S/N values <0.60 were considered positive for antibodies to influenza A virus.

Hemagglutination-inhibition (HI) test

Due to limited availability of the required reagents, representative sera samples that were positive for influenza A virus antibodies by NP-ELISA were selected ($n=35$) for the HI assay in order to determine the influenza A virus subtype.

Treatment of sera with a receptor-destroying enzyme (RDE): To remove nonspecific hemagglutinin, test sera were treated with RDE (Influenza Reagent Resources (IRR), Centers for Disease Control and Prevention (CDC), U.S.A.)

according to published methods [6, 11, 25] with some modifications. RDE was reconstituted with physiological saline according to the provided instructions; 0.9 ml of RDE was mixed with 0.3 ml of test serum and incubated overnight at 37°C in a water bath. After overnight incubation the mixture was heated at 56°C for 30 min to inactivate remnants of RDE in the treated sera. Serum samples were allowed to cool to room temperature and 1.8 ml of normal saline was added to give a final dilution of 1:10.

Hemadsorption with chicken red blood cells (RBCs): Each RDE-treated sample was added to packed RBCs in centrifuge tubes at a ratio of 20:1 and thoroughly mixed and then incubated at 4°C for 1 hr, with intermittent mixing to resuspend red cells. The red cells were allowed to settle by gravitation and the supernatant was collected into cryovials and used for HI testing.

The test was performed as described by Hsiung [13] with some modifications using a U-bottom microtitre plate by first carrying out hemagglutination (HA) to appropriately determine 4HA units of the H3N2 and H7N3 antigens used (OIE/FAO reference laboratory, Istituto Zooprofilattico Sperimentale delle Venezie, Padova, Italy). The HI test targets cross-reactivity of hemagglutinin protein of avian H7 and H3 origin. Treated sera were diluted serially in twofold with PBS while 4HA units each of reconstituted antigens for H3 and H7, which had been predetermined was added, and the plate was shaken and incubated at room temperature (27°C) for 30 min to allow the reaction to take place. Pooled chicken RBCs (1% suspension) were added and incubated for 30 min room temperature (27°C). The test was read by tilting the microtitre plate at an angle to observe RBCs streaming at the bottom of the well. The HI titre was taken as the highest dilution of serum with complete inhibition of agglutination in a twofold serial dilution of the original sera.

Results

Data regarding the age range (2–22 years), sex, breeds and vaccination history of horses sampled from different stables (designated A–G) were recorded (Table 1). A total of 284 serum samples collected from 7 stables in Kaduna metropolis were analyzed. One hundred and seventy-three (60.9%) tested positive for antibodies against EIV A by NP-ELISA. Stable B had the highest seroprevalence (85.7%) and stable C had the lowest (27.3%). Thirty-five representative ELISA reactors were randomly selected for HI assay and the results are presented in Table 2. EIV A subtypes H3 and H7 were detected in the horse stables in Kaduna metropolis. The results show that the H3 subtype was more prevalent with 21 (60%) of the tested sera positive with an HI titre of 3 log₂ (8–256), while 7 (20%) had an HI titre of 3 log₂ to 4 log₂ (8–16) for the H7 subtype.

Table 1. Detection of influenza A virus antibodies by ELISA in horse stables in Kaduna metropolis, Nigeria

Stable	No. of samples	Breed	Sex		Age (years) range	Vaccination history	EI A ELISA positive (%)
			Male	Female			
A	17	Mixed*	10	7	4–10	NV	14 (82.3)
B	28	Exotic	12	16	2–9	NV	24 (85.7)
C	22	Local	15	7	6–22	NV	6 (27.3)
D	93	Exotic	22	71	3–13	NV	46 (49.5)
E	15	Exotic	3	12	3–11	NV	11 (73.3)
F	17	Local	16	1	4–15	NV	11 (64.7)
G	92	Local	22	70	2–17	NV	61 (66.3)
Total	284						173 (60.9)

*10 exotic and 7 local breeds; NV: not vaccinated.

Table 2. EIV subtypes distribution in horse sera tested in Kaduna metropolis by the hemagglutination inhibition test

Subtypes	HI titre-twofold sample dilution								Total
	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	
H3	-	-	2	3	4	5	3	4	21
H7	-	-	5	2	-	-	-	-	7

Discussion

The ELISA results in this report show that 173 (60.9%) of the horses in the 7 tested stables in Kaduna metropolis were positive for antibodies against EIV A. Further analysis on some randomly selected ELISA reactors (n=35) with an HI assay revealed the presence of EI A subtypes H3 (n=21) and H7 (n=7). Seven ELISA-positive sera had an antibody titre of less than 1 log₂ for both H7 and H3 in the HI assay, which may not be unrelated to the high sensitivity of the ELISA compared with HI. However, these samples could be tested for other HA subtypes when available to our laboratory. The detection of EI A virus antibodies by two independent serological tests is an indication of EI infection past or present in the stables sampled. EIV subtypes H3 and H7 have been previously reported among polo horses in south western region of Nigeria [4, 28]. The diagnosis of EIV could be based on virus isolation in embryonated eggs from horses with acute infection. However, the demonstration of an antibody response is an evidence of subclinical infection. Due to limited laboratory resources, we were only able to select a few ELISA-positive samples for HA to determine prevailing subtypes. Test sera were first treated with RDE to remove nonspecific inhibitors according to the OIE protocol. Other limitations in this study include the possibility that RDE does not always inactivate nonspecific inhibitors that may occur at low levels in horse sera [6]. Though potassium periodate is recommended as the best destroyer of nonspecific inhibitors, but it was not available

to us. Also lacking were the equine strains of H3 and H7 antigens, which is why we used avian strain subtypes. However by including appropriate internal positive and negative controls we sought to validate both the ELISA and HI tests. Furthermore, cross-reactivity of antigens and antisera has been demonstrated in influenza serology and the species in which antibodies or antigens are raised influences the degree of cross-reactivity. Given that all influenza A subtypes (H1–16) have avian progenitors the HI test was able to detect cross-reactive subtype-specific activities at low levels especially for H7. Nevertheless, the HI titre could probably be higher if species-specific strains H7N7 (equine-1) and H3N8 (equine-2) were used as antigens in the assay.

This study is significant in this field because of its use of a large number of horses and because it shows the probability of two EIV strains circulating in this part of Nigeria, which requires further investigation. Equine Influenza could have deleterious health and economic consequences for the boisterous equestrian activities in the country.

The epidemiology of EI in Nigeria could be linked to importation of horses from EIV endemic countries as previously described in south western Nigeria in 1991 amongst polo horses [2]. Molecular studies on the three H3N8 subtypes independently isolated shows that their genes are equine in origin and that the hemagglutinin (HA) glycoprotein is most closely related to European and Scandinavian isolates [3]. Although there has been no EIV outbreak reported in Nigeria since 1991, serological evidence of the disease has been demonstrated in horses and donkeys in

some parts of the country [4, 28].

In countries like South Africa and India where major epidemics have been recorded in the past, infections were introduced by the importation of sub clinically infected horses from Europe and America as confirmed by the close antigenic relationships with isolates from those countries [16]. Polo tournaments and other equestrian activities are popular in Nigeria often attracting horses from far and near some of which are imported and kept near together with large numbers of human spectators. Though natural human infection with equine influenza is rare, experimental infection of human volunteers with equine influenza virus has been demonstrated [14]. A study has also shown an antigenic relationship between the equine and the Hong Kong variants of the influenza subtype H2 virus [15]. Vaccination against EI is an effective method of disease control in developed countries; however, the importation of unregulated horses in conjunction with inadequate quarantine procedures could result into major outbreaks through the introduction of exotic viral strains with significant health and economic consequences. Other animal influenza viruses particularly swine [22] and avian [23] influenza viruses, have been reported at the human-animal interface in Nigeria. Therefore, the risk of exposure and virus reassortment of the equine, swine, avian and human influenzas if and where they co-circulate is a cause for concern particularly because of the existing poor level of biosecurity. There are no national surveillance programs for EIV in Nigeria predominantly due to lack of funding. Furthermore, only partial serological data are available for EIV in Nigeria, and serological testing is usually only performed for a specific location and specific research studies. It is therefore necessary to put in place an EI national surveillance plan with recommendations for an appropriate control program to safeguard the large population of horses in Nigeria.

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