Epidemiol. Infect. (1996), **117**, 367–374

Antigenic and genetic analysis of equine influenza viruses from tropical Africa in 1991

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(*Accepted 23 May 1996*)

SUMMARY

A detailed analysis of equine (H3N8) influenza viruses isolated in Nigeria during early 1991 has been undertaken. Antigenic analysis and the complete nucleotide sequence of the HA gene of three Nigerian equine influenza viruses A/eq/Ibadan/4/91, A/eq/Ibadan/6/91 and A/eq/Ibadan/9/91 are presented and limited sequence analysis of each of the genes encoding the internal polypeptides of the virus has been carried out. These results establish that, despite the geographical location from which these viruses were isolated, two were similar to the viruses which were concurrently causing disease in Europe in 1989 and 1991 and were related to viruses that have been predominating in horses since 1985. The third was more closely related to viruses isolated from 1991 onward in Europe but also in other parts of the globe. A comparison of the nucleotide sequence of two of the viruses isolated in Nigeria (A/eq/Ibadan/4/91 and A/eq/Ibadan/6/91) with a European strain (A/eq/Suffolk/89) showed limited variation in the haemagglutinin gene which caused amino acid substitutions in one of the antigenic sites: this mutation resulted in the potential production of a new glycosylation site in antigenic site A. The other Nigerian virus (A/eq/Ibadan/9/91) showed only a single one amino acid change from another European strain (A/eq/Arundel/12369/91). The two distinct Nigerian viruses had several amino acid substitutions in the antigenic sites of the haemagglutinin glycoprotein.

INTRODUCTION

The first isolation of equine influenza virus in tropical Africa has recently been reported [1]. Three viruses were isolated following an outbreak of equine influenza in polo horses in Ibadan, Nigeria in January 1991 and were shown to be antigenically H3N8, members of the equine-2 sub-type of influenza viruses. There has been a reemergence of equine H3N8 viruses in recent years in South Africa [2, 3]; Italy [4]; India [5]; the United Kingdom [6]; Scandinavia [7] and China [8, 9].

The epidemiological and evolutionary relationships between equine influenza viruses have been examined by phylogenetic analysis of the nucleotide sequence of the haemagglutinin gene of these viruses and distinct conclusions have been drawn. An analysis carried out by Endo and colleagues [10] suggested that there were two main evolutionary lineages of virus while Kawaoka and colleagues [11] adduced that there was only one. More recent analyses of equine H3 viruses, which includes analysis of additional viruses isolated in 1989 and 1991, concluded that there has been a change in evolutionary pattern: prior to 1984 no clear linear relationship between nucleotide difference and time of isolation was apparent but following 1984 a linear relationship was observed [12]. In contrast, the

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viruses isolated in China during 1989 and 1990 had a distant phylogenetic genetic relationship to typical equine HA genes and, furthermore, the genes which encode the internal polypeptides were derived from virus strains associated with birds rather than horses [8] but they had been superceded by 'equine' H3N8 viruses during the 1993–4 epidemic of equine influenza in China [9, 13].

In light of this background, an antigenic analysis and the complete nucleotide sequence of the HA gene of two Nigerian equine influenza viruses, and limited sequence analysis of each of the genes encoding the internal polypeptides, has been carried out. These results establish that they were most similar to the viruses which were concurrently causing disease in Europe and were sampled from the same evolutionary tree of viruses that has been predominating since 1985.

MATERIALS AND METHODS

Virus isolation and growth

The virus isolation and preparation of virus was done in 11-day old embryonated hen's eggs at 35° for 48–72 h [1].

Haemagglutinin inhibition (HI) assays

HI assays were carried out by a standard methods [14] using a panel of 18 monoclonal antibodies raised against A/equine/Miami/1/63, A/equine/Fontainebleu/1/79, A/equine/Tennessee/5/86 and A/equine/Kentucky/81 (provided by Dr R. Webster, St Jude Children's Research Hospital, Memphis, TN., and Mr A. Douglas, National Institute for Medical Research, Mill Hill, London, UK).

RNA amplification and sequence analysis

Chick embryo fibroblast cells were infected with virus (> 10 pfu per cell) and infected cell RNA prepared 8 h post infection [15]. Influenza virus vRNAs in the infected cell RNA preparation were amplified in a single multiplex PCR reaction [16]. Partial DNA sequencing of amplified cDNA corresponding to each vRNA segment was carried out using radiolabelled segment specific oligonucleotides and cycle sequencing as described previously [16], and the results analysed using a computer program for best local homology searching on a Massively Parallel Processor [17]. The complete HA gene sequence was determined on the same multiplex PCR product using radiolabelled oligonucleotides and also on the HA1 coding region for A/eq/Ibadan/9/91. Oligonucleotides were made on an Applied Biosystems 381A synthesizer or were a gift of Dr R. Webster, St. Jude Children's Research Hospital, Memphis, TN.

DNA sequence and phylogenetic analyses

Nucleotide sequence analysis was carried out using the University of Wisconsin GCG package of computer programs [18]. Phylogenetic analysis was done by the Distances, Neighbor-Joining and DNAPARS programs of the PHYLIP package [19].

RESULTS

HI analysis of Nigerian equine influenza viruses

Three equine influenza viruses isolated in hens eggs following an outbreak of respiratory disease in Ibadan, Nigeria, were examined by HI analysis using monoclonal antibodies raised against four equine influenza viruses. All three Nigerian viruses gave generally similar patterns of reactivity to each other although some variation was seen (Table 1). The significance of the variation observed is not clear but differences in the titres of HI of 2-, 4- and 8-fold are generally considered to be of minor importance. The three virus isolates cannot, therefore, be considered identical but are likely to be closely related. Parallel tests were not carried out with the laboratory strains against which the monoclonal antibodies were made because of the risk of cross contamination in the subsequent studies which involved PCR. Previously, it has been shown that equine H3 viruses differred antigenically [11] but viruses isolated from 1983–7 (the latest example taken) regained reactivity to monoclonal antibodies to the prototype virus (Miami/63). The Nigerian viruses also reacted with monoclonal antibodies directed against viruses isolated during 1963, 1979, 1981 and 1986. Although, in this work, the panel of monoclonal antibodies used was not identical to that used by Kawaoka and colleagues [11], the broad cross reactivity suggests that these African viruses were similar to those isolated in USA between 1983 and 1987.

The origin of the genes of the virus non-glycosylated polypeptides

Partial nucleotide sequences were obtained from cDNAs corresponding to RNA segments 1, 2, 3, 5, 7 and 8 of A/eq/Ibadan/4/91 and A/eq/Ibadab/6/91

Table 1. *HI titres of monoclonal antibodies against three equine influenza viruses from tropical Africa*

Monoclonal antibody	Miami/1/63*				Fbleu/1/79*		Tennessee/5/86†			Miami/1/63†				Kentucky/81†			
Clone name	H1	H3	H4	H6	H1	H2	2/1	4/1	14/1	22/1	EQ1	EQ5	EQ6	EQ7	1/1	2/1	3/1	4/1
Virus																		
IBADAN/4/91	5120	5120	2560	2560	640	640	5120	320	2560	640	80	80	80	40	1280	320	40	80
IBADAN/6/91	5120	2560	5120	5120	2560	2560	5120	1280	2560	2560	640	640	640	40	2560	2560	320	320
IBADAN/9/91	2560	1280	640	5120	5120	1280	1280	160	320	320	160	160	80	160	320	320	80	80

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by use of a rapid method for the characterization of influenza virus genes based on multiplex PCR and cycle sequencing [16]. The partial cDNA sequences (100–147 bases) obtained were compared with the virus DNA sequences in the EMBL sequence library. The results from the database comparison are summarized in Table 2.

The results showed that for segments 1, 2, 5 and 7 the closest related sequence was from equine H3 viruses isolated during 1986 (equine/Tennessee/1986 for segments 2 and 7 and equine/Kentucky/86 for segment 1; segment 5 was equally related equally to each of the 1986 viruses). The nucleotide sequence for segment 3 was most closely related to an equine-1 virus (London/1416/73); but it is known that there had been genetic shift in the internal genes of equine H7N7 viruses in which those of the RNP complex were 'usurped' by those of the H3N8 sub-type [16, 20–23]. RNA segment 8 was most similar to an H3N8 virus from 1976.

Overall, these findings on the relationship of these African viruses group them with viruses previously present in the Western Hemisphere, but the results do not imply identity with the strains studied previously for which nucleotide sequence data is available.

Sequence analysis of the haemagglutinin gene

The nucleotide sequence of the complete HA gene of each of the three Nigerian isolates was determined on RNA segment 4 amplified in a multiplex PCR procedure [16], and, for A/eq/Ibadan/9/91, additionally on an amplified region of the genome encoding the signal sequence and the HA1 polypeptide-chain, all without molecular cloning. The PCR product was sequenced directly by cycle sequencing using 5′ terminal end labelled oligonucleotide primers. In our experience, cycle sequencing PCR products obviates the necessity to determine the nucleotide sequence of multiple cDNA clones obtained following PCR which is required with the alternative strategy of cloning PCR products since, as with direct RNA sequencing, an average sequence is obtained as long as sufficient RNA was used to make the cDNA copy prior to PCR.

The nucleotide sequences of the HA gene of A/eq/Ibadan/4/91 and A/eq/Ibadan/6/91 show no variation in the consensus nucleotide sequence although the nucleotide sequence of the HA gene of A/eq/Ibadan/9/91 was different. The nucleotide sequences have been deposited at the EMBL nucleotide sequence database at the European Bioinfor-

https://doi.org/10.1017/S0950268800001552 Published online by Cambridge University Press

Table 2. *Homology scores of EQ/Ibadan/4/92 with other influenza viruses*

	Probe sequence	Match no.	Virus matched	Match	Mismatch	Homology (%)
Segment 1	48–153	1	A/Eq/KY/2/86 (H3N8)	105	1	99·1
		2	A/Eq/Lond/1416/73 (H7N7)	103	3	97·2
		3	A/Mallard/NY6750/78 (H2N2)	100	6	95·2
Segment 2	63–173	1	Eq/Tenn/5/86 (H3N8)	110	1	99·1
		2	Eq/Lond/1416/73 (H7N7)	109	2	98·2
		3	Swine/Ger/2/81 (H1N1)	103	8	92·8
Segment 3	64–194	1	Eq/Lond/1416/73 (H7N7)	129	1	98·5
		2	Eq/Tenn/5/86 (H3N8)	126	4	96·2
		3	WSN/33 (H1N1)	119	8	93·0
Segment 5	48–195	1 =	Eq/Tenn/5/86 (H3N8)	143	5	96·6
		1 =	Eq/KY/2/86 (H3N8)	143	5	96·6
		3	Eq/Lond/1416/73 (H7N7)	141	7	95·3
		4	Eq/Miami/1/63 (H3N8)	137	11	93·8
		5	Gull/MD/5/77 (H11N9)	129	18	87·8
Segment 7	34–174	1	Eq/Tenn/5/86 (H3N8)	141	0	100
		2	Eq/Ky/2/86 (H3N8)	140	1	99·3
		3	Mallard/NY/6750/78 (H2N2)	139	2	98·6
Segment 8	27–173	1	Eq/NMKT/76 (H3N8)	144	2	99
		2	Eq/Miami/1/63 (H3N8)	141	5	97
		3 =	Mynah/Thai/76 (H3N1)	140	6	96
		3 =	Duck/Eng/56 (H11N6)	140	6	96

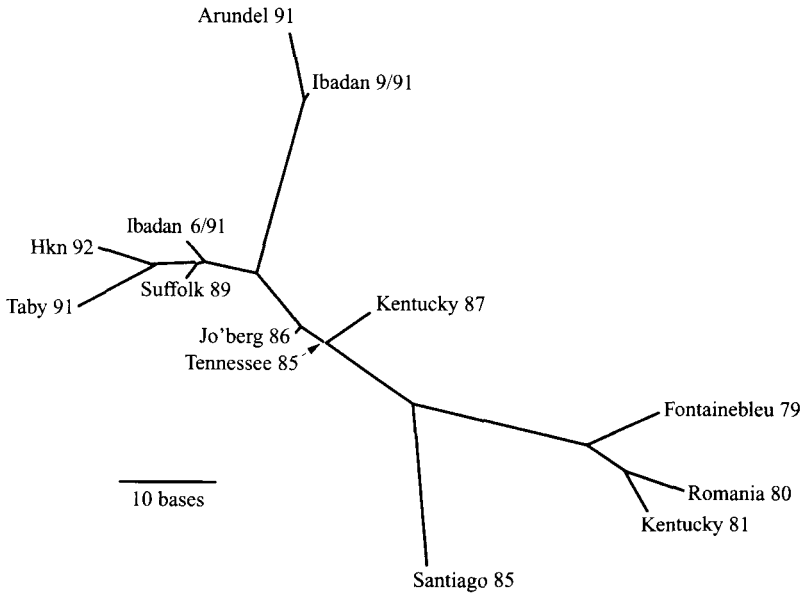

Fig. 1. Cluster analysis of the nucleotide sequence of A/equine/Ibadan/6/91 with representative strains of equine influenza viruses. Sequence differences were estimated using DNADIST and the phylogenetic tree produced using Neighbor-Joining [19]. The published sequences are: Fontainebleu 79, A/eq/Fontainebleu/79, [32]; Romania 80 A/eq/Romania/80; Kentucky 81, A/eq/Kentucky/81, Santiago 85, A/eq/Santiago/85; Tennessee 85, A/eq/Tennessee/85; Kentucky 87, A/eq/Kentucky/87, [11]; Jo'berg 86, A/eq/Johannesburg/86 [3]; Taby 91, A/eq/Taby/91 [7]; Suffolk 89 A/eq/Suffolk/89 [26] and Hkn 92, A/eq/Hong Kong/92 [27] and A/Arundel/12369/91 [24].

matics Institute, Hinxton, UK (World Wide Web address: http://www.ebi.ac.uk; email: datalib@ebi.ac.uk) and have been given the accession numbers: A/equine/Ibadan/6/91 X95637; A/equine/Ibadan/9/91, X95638.

These DNA sequences, and those of other equine H3 haemagglutinins, were analysed by cluster analysis with the DNADIST program and a Neighbor-Joining tree was derived [19]. A portion of this tree is shown in Figure 1 and demonstrates a close relationship

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Table 3. *Nucleotide sequence variation between UK and Nigerian equine influenza viruses*

Base number	Suffolk 89 [25]	Ibadan 6/91	Ibadan 9/91	Arundel 91 [24]	Amino acid sequence change	Remarks
45	A	G	A	A	I > V	Signal peptide, position 6
83	C	C	T	C	Synonymous	—
245	G	G	A	A	Synonymous	—
308	T	T	C	C	Synonymous	—
361	A	A	G	G	N 96 S	HA1 antigenic site A
428	A	A	G	G	Synonymous	—
478	G	C	G	G	R 135 T	HA1 antigenic site A, potential N-linked glycosylation at N133
486	G	G	T	T	A 138 S	HA1 antigenic site A
540	A	A	G	G	K 156 E	HA1 antigenic site B
551	T	T	C	C	Synonymous	—
562	T	T	C	C	I 163 T	HA1 antigenic site B
599	T	T	C	C	Synonymous	—
658	T	T	C	C	Synonymous	—
693	G	G	A	A	E 207 K	HA1 antigenic site D
711	G	G	A	A	V 213 I	Buried
800	T	T	C	C	Synonymous	—
805	C	T	T	T	T244 M	T unique to Suffolk 89
824	C	C	T	C	Synonymous	—
856	G	G	A	A	R 261 K	HA1 antigenic site D/E
901	C	C	T	T	T 276 I	HA1 antigenic site C
943	A	A	A	G	N 290 S	HA1 antigenic site C
965	G	G	A	A	Synonymous	—
1028	C	T	T	T	Synonymous	—
1168	C	C	G	ND	A 36 G	HA2
1188	G	A	G	ND	A 43 T	HA2 unique to Ibadan 4/91 and 6/91
1271	C	C	T	ND	Synonymous	—
1298	T	T	C	ND	Synonymous	—
1322	C	C	T	ND	Synonymous	—
1430	G	G	A	ND	Synonymous	—
1571	A	A	G	ND	Synonymous	—

between the viruses isolated in Nigeria and those isolated in Europe during 1989 and Europe and elsewhere during 1991, although A/eq/Ibadan/6/91 and A/eq/Ibadan/9/91 cluster in distinct lineages of these recently divergent clades [24].

This relationship is reflected in a comparison of the amino acid sequence changes which have occurred in the tropical African and other equine H3 viruses. In Table 3 the differences in base and amino-acid sequence between the 1989 virus, A/eq/Suffolk/89 [25], a representative example of a virus from a second branch of the Neighbor-Joining tree (Arundel/12369/91, [24]) and the Nigerian viruses are shown.

Only five base differences are seen between Suffolk/89 and A/eq/Ibadan/6/91, all but one of which result in amino acid substitutions. One, at position 135 of HA1 (R → T), results in the introduction of a potential new site for N-linked glycosylation at amino acid 133. The other amino acid substitutions are in the signal sequence, in HA2 and one at position 244. Only the region of the genome encoding the signal peptide, HA1 and the amino-terminal 40 amino acids of HA2 has been determined for the strain Arundel/12369/91. There are three base changes in that region of the genome between Arundel91 and Ibadan/9/91; two were synonymous, encoding the same amino acid residue, and one encodes an amino acid substitution in antigenic site C. Between A/eq/Ibadan/6/91 and A/eq/Ibadan/9/91 27 base differences were observed: 15 were synonymous and 12 lead to amino acid

https://doi.org/10.1017/S0950268800001552 Published online by Cambridge University Press

substitutions. One amino acid substitution was located in the signal peptide, and two were in HA2. All but one of the other amino acid substitutions were located in, or close to, each of the antigenic sites of the haemagglutinin, with three changes seen in site A, two in site B, one in site C, one in site D and one substitution that was located at the junction of sites D and E. The single amino acid substitution in HA1 outside the antigenic sites (residue 213) is not exposed on the surface of haemagglutinin trimer.

DISCUSSION

We have recently described only the third equine influenza virus that has been isolated from the African continent but the first from tropical Africa [1]. This paper characterizes the viruses at the molecular level by examining the nucleotide sequence of the virus genes and by the antigenicity of the haemagglutinin.

Nucleotide sequence analysis of the RNAs encoding the genes of the ribonucleoprotein complex and the non-structural proteins demonstrated that these genes are most closely related to those of the equine H3N8 viruses of the western hemisphere rather than to the H3N8 virus isolated from horses in Northern China in 1989 and 1990 which was related to viruses isolated from birds [8]. The partial nucleotide sequences of the new African virus differed from those of other equine influenza viruses but in all cases, except RNA segment 3, the closest relationship of nucleotide sequence was to a virus isolated from the USA during 1986. RNA segment 3, on the other hand, was most closely related to a virus isolated in London in 1973. However, an insufficient number of equine influenza virus genes are available in sequence databases to enable firm conclusions to be drawn about any potential epidemiological significance of the origins of the genes of the ribonucleoprotein complex and the non-structural proteins of this tropical African virus, other than that they are of equine-2 sub-type in origin, rather than being related to viruses from birds or other animals.

The antigenicity of the haemagglutinin was examined to establish the relationship of the HA of the African viruses to each other. Of 18 monoclonal antibodies studied all displayed some reactivity to each of the three viruses tested. This is not unexpected, since, although antigenic variation has been recorded since the first isolation of an equine-2 sub-type of virus during 1963, viruses isolated in the USA in the mid- to late-eighties demonstrated sensitivity to HI by monoclonal antibodies raised against the prototype virus whilst other strains isolated in the nineteen-seventies and in 1981 and 1982 did not [11]. The pattern of sensitivity could reflect a close relationship between the USA equine viruses of the mid and late eighties and the new African viruses described here. However, this conclusion would not differentiate between an American or South African origin for the Ibadan viruses, since the HA gene of the virus isolated from Johannesburg in 1986 differed from one isolated in Kentucky during 1986 by only two bases and one amino acid [3].

Phylogenetic analysis of the HA gene shows that the Nigerian viruses are most closely related to viruses isolated contemporarily in Northern Europe, Hong Kong and North and South America (Fig. 1) [24]. These viruses, which exist in two phylogenetic groups, are considered to result from antigenic drift from the earlier strains isolated during the eighties [7, 12, 25, 26]. This antigenic drift, as opposed to genetic drift, is manifested by an increase in the number of amino acid changes in the antigenic sites of the molecule that become fixed. Two of the viruses isolated from Nigeria (A/eq/Ibadan/6/91 and A/eq/Ibadan/4/91) viruses show only four amino acid changes from the best characterized Northern European strain of virus (Suffolk/89), shown in (Table 3). One of the amino acid substitutions maps to an antigenic site [27]; the others are within the signal sequence, in a region of HA1 not thought to be antigenic, and in HA2. The third new isolate from Africa, A/eq/Ibadan/9/91, belonged to another phylogenetic lineage comprising isolates from Europe and elsewhere. Only a single amino acid change was demonstrated (in antigenic site C) when the sequence of the HA1-encoding portion of the HA-glycoprotein gene was compared to A/eq/Arundel/12369/91 [24] but in other examples of viruses in the same phylogenetic lineage, this amino acid residue was asparagine like Ibadan/9/91 [28]. Between the distinct Nigerian isolates there is considerable genetic variation, with amino acid substitutions in each of the antigenic sites. The change at position 135 in A/eq/Ibadan/6/91 and A/eq/Ibadan/4/91 introduces a new N-linked glycosylation site at position 133 (NGR → NGT) although this site is not present in other recently isolated equine influenza viruses in this phylogenetic lineage. We have no direct evidence for its use but the mobility of the haemagglutinin in SDS gels of both A/eq/Ibadan/4/91 and A/eq/Ibadan/6/91 is distinct from that of A/eq/Ibadan/9/91 (data

not shown). The utilization of the new glycosylation site may be significant antigenically since it is analogous to the observation of the aquisition by an epidemic strain of human influenza virus (A/England/878/69) of a new glycosylation site (at position 63) observed by Skehel and colleagues [29] and was also in a variant selected by a monoclonal antibody which affects their antigenicity. The ratio of 14 amino acid substitutions in total (10 of which are in antigenic sites) to 16 'silent' base changes cannot be taken as direct proof of antigenic drift having occurred as a result of natural selection, however. The low ratio of non-synonymous-to-synonymous mutations seen here (14–16, respectively) may be caused by strong constraints to variation within the regions of the haemagglutinin glycoprotein that are not exposed in its three dimensional structure, for example. If variation was totally random then the ratio of non-synonymous to synonymous would be 3:1.

A possible cause of differences between closely related virus isolates is selection of variants during isolation, usually in hens' eggs [30]. The possibility of selection imposed during isolation of equine influenza viruses has been examined [31] and selection of variants appeared to be imposed by passage of virus in tissue culture rather than in eggs. The variant amino acids seen between two Ibadan (4/91 and 6/91) viruses (both isolated in hens' eggs) and the Suffolk virus were at positions 135 and 244 of HA1 and position 43 of HA2, none of which have been described as sites that are subject to selection during isolation of human H3 viruses in eggs [30]. Likewise, the single amino acid difference observed between Ibadan/9/91 and Arundel/91 (at position 290) has not been a residue involved in selection in human H3 viruses. Amino acid residues 138 and 156 of HA1 have been associated with egg adaptation of human virus strains and these residues differ between the amino acid sequences of A/eq/Ibadan/6/91 (& A/eq/Ibadan/4/91) and A/eq/Ibadan/9/91: these amino acid substitutions similarly, therefore, may reflect selection during the isolation of the African viruses. Other changes between the African influenza viruses are likely to be differences present in the original viruses in the infected ponies but the possibility that selection during isolation has resulted in some of the heterogeneity observed between these strains of virus cannot be totally discounted.

Recent outbreaks of equine influenza in Hong Kong duirng 1992 were epizootologically related to contemporary outbreaks in Europe [26] whereas the epizootology of viruses those from mainland China isolated during 1993/4 is less clear [13]. Although in Nigeria, there are fewer opportunities for introduction of virus from abroad than in Hong Kong, which has numerous links with the bloodstock industry of the middle east and the western hemisphere, ponies are imported into Nigeria for use in polo are from Argentina and UK. In late 1990 it was known that 10 horses were brought to Ibadan in January 1991 for the annual polo tournament. It seems likely that these few imported animals carried the viruses isolated during the outbreak that we have described [1].

The simultaneous isolation of equine influenza viruses of two phylogenetically distinct groups in January 1991 in Ibadan, Nigeria during an outbreak of severe illness at a polo tournament reinforces the importance of global monitoring of equine influenza viruses; it demonstrates again the rapid spread of influenza viruses around the world and highlights the risks that accompany transport and crowding of horses at tournaments, race meetings or markets.

ACKNOWLEDGEMENTS

We thank Dr C. Bostock for a critical review of the manuscript. This work was first presented at the IXth International Congress of Virology, 1993, Poster: P15-6. CAOA was in receipt of a Commonwealth Universities Fellowship whilst on leave of absence from University of Ibadan, Nigeria.

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