Research Article

Nucleotide and Amino acid changes map to Functional Domains on the Haemagglutinin of A/equine/Ibadan/4/91 (H3N8) influenza virus

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ABSTRACT: The application of the method of rapid sequencing of equine influenza A virus A/equine/Ibadan/4/91 (H3N8) haemagglutinin (HA) gene in ELISA plates is reported. There was no nucleotide change compared with the sequence we earlier obtained for this virus by cycle sequencing which indicates that the present method is equally sensitive and specific but there was only one nucleotide change at position 478 compared to A/eq/Ibadan/6/91 (H3N8) isolated at the same time. Compared with prototype European strain, A/eq/Suffolk/89 (H3N8), nucleotide and amino acid changes observed in the HA of A/eq/Ibadan/4/91 mapped to functional domains of the molecule: signal peptide and antigenic sites. Nucleotide changes occurred at positions 45 (A→G), 478 (G→C), 562 (T→G), 805 (C→T) in HA1 and 1188 (G→A) in HA2 while amino acid changes occurred at residues 6 (I→V) in the signal peptide, 135 (R→T), 178 (I→T), 244 (T→M) in the HA1 and 43 (A→T) in the HA2. The change at residue 135 introduced a new potential asparagine-linked glycosylation site at residue 133. The genetic and antigenic implications of these changes are highlighted. The specificity and advantages of the method used over reverse transcription-polymerase chain reaction (RT-PCR) and cycle sequencing of PCR products are discussed.

Keywords: *nucleotide sequence analysis, ELISA plates, functional domains.*

INTRODUCTION

Influenza viruses are enveloped, negative-sense, single-stranded RNA viruses with segmented RNA genome. They belong to the family *Orthomyxoviridae* (Palese and Shaw, 2007). There are five general: *Influenzavirus A*, *InfluenzavirusB*, *Influenzavirus C*, *Thogotovirus* including Thogoto and Dhori viruses, and *Isavirus* including infectious salmon anaemia virus (Kawaoka *et al.*, 2005). Influenza A viruses are however further classified into subtypes based on the antigenicity of their surface haemagglutinin (HA) and neuraminidase (NA) molecules. Currently, there are 16 HA (H1-H16) and nine (N1-N9) subtypes (Fouchier *et al.*, 2005, Wright *et al.*, 2007). The virus particle contains a lipid bilayer in which are anchored the HA, NA and a transmembrane pH gated proton ion channel, matrix 2 (M2) proteins. The matrix 1 (M1) protein forms a layer beneath the envelope inside which are encapsidated the basic protein 2 (PB), basic protein 1 (PB1), acidic protein (PA), HA, nucleoprotein (NP), NA, M and the non-structural (NS) genes. The genome of influenza A and B viruses is divided into eight single-stranded RNA gene segments which are packaged as ribonucleoprotein complex while that of serotype C is divided into seven, lacking the NA gene segment (Palese and Shaw, 2007).

Influenza B and C viruses infect human hosts while influenza A viruses infect a wide range of mammals and birds in diverse geographical locations (Webster *et al.*, 1992). The most commonly affected host species

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are man, pigs, horses, poultry, sea mammals, wild birds (Webster *et al*., 1992, Adeyefa and McCauley, 1994, Adeyefa, *et al*., 1994) and, more recently, the big cats, tiger, leopard, as well as domestic cats and dogs (Keawchareon *et al*., 2004, Kuiken *et al*., 2004, Crawford *et al*., 2005, Tiesin *et al*.,2005, Webster *et al*., 2006) and migratory waterfowls which serve as their reservoir (Webster *et al*., 1992, Lin *et al*., 2005). Influenza A viruses, because of their segmented genomes, can undergo antigenic drift through point mutations (Wiley *et al*., 1981) and genetic shift in which the virus genes undergo reassortment/genetic recombination and a novel virus is generated which can replicate in new host species (Webster *et al*., 1992, Wright *et al*., 2007). There is also interspecies transmission of influenza A viruses. Well known examples of animal and avian viruses infecting man include infection of man with swine viruses (Kendal *et al*., 1977, Rota *et al*., 1989), avian H5N1 viruses infecting man in Hong Kong in 1997 with fatality (Bender *et al*., 1998) and more recently widespread outbreaks of highly pathogenic avian influenza (HPAI) H5N1 viruses in Southeast Asia, Europe, the Middle East and Africa (Shortridge *et al*., 1998, Subbarao *et al*., 1998, Mase *et al*., 2004, Peiris *et al*., 2004, Webster *et al*., 2006, Wright *et al*., 2007) in which over 107 individuals have died including at least one in Nigeria (Mase *et al*., 2005, Webster *et al*., 2006, Nasidi *et al*., 2007, Wright *et al*., 2007) and over 140 million domestic birds have died or were culled with serious economic losses to the poultry industry worldwide (Monne *et al*., 2006, Webster *et al*., 2006, Wright *et al*.,2007, Aiki-Raji *et al*., 2008, Cattoli *et al*., 2009). There are also reports of animal and avian viruses infecting animals, examples of which include swine-like viruses isolated from ducks and turkeys (Hinshaw and Webster, 1982, Ludwig *et al*., 1994), avian-like H1N1 viruses infecting pigs (Schultz *et al*., 1991), avian H3N8 virus infecting horses with high morbidity and mortality in southern China (Guo *et al*., 1992), an alarming recent transmission of equine H3N8 virus to greyhounds in the USA with a high morbidity of haemorrhagic pneumonia (Crawford *et al*., 2005) and also viruses from other species which have undergone genetic reassortment being isolated from pigs (Castrucci *et al*., 1993). The most recent of interspecies transmission was the triple reassortant swine-origin H1N1 2009 virus infecting pigs and man with fatality in Mexico and USA. It resulted inhuman-to-human transmission with worldwide spread to over 111 countries,>1,630,000 confirmed cases and > 17,500 deaths including at least one in Nigeria,and in June, 2009 was declared the first pandemic of the 21st century by the WHO (World Health Organisation by which

time the virus had become known as pandemic H1N1 2009 virus. The mechanisms involved in the generation of novel viruses include point mutations, deletions and insertions in the virus genes and recombination in the gene segments (Webster *et al*., 1992, Monne *et al*., 2008, Owoade *et al*., 2008, Cattoli *et al*., 2009).

In the light of the ability of influenza A viruses to infect new host species, their potential for rapid global spread and the serious economic losses associated with their infection and disease, knowledge of the precise origin of the virus genes as an important characteristic of the virus is desirable. A number of techniques have been described for analyzing influenza virus genes (Adeyefa, 1995, Adeyefa *et al*., 1994, 1996, Adeyefa and McCauley, 1997a, Offringa *et al*., 2000, Storch, 2007). Similarly, rapid diagnosis and identification of circulating viruses are of paramount importance in order to institute effective control strategies (Adeyefa, 1996). A number of diagnostic methods have also been described including immunoassays, electron microscopy and molecular methods (Adeyefa, 1996, Adeyefa and McCauley, 1994, 1997, Adeyefa *et al*., 1994, 1996, Offringa *et al*., 2000, Storch, 2007, Wright *et al*., 2007). However, the most reliable method of identification and characterization of influenza viruses is genetic analysis. We have previously described a rapid method for partial nucleotide sequence analysis of the RNA genes of various influenza A virus subtypes isolated from man, pigs, horses and poultry in ELISA plates (Adeyefa and McCauley, 1997a). We have now used this method to determine the complete nucleotide sequence of the HA gene segment of A/equine/Ibadan/4/91 (H3N8) designated Ib4, earlier isolated in Ibadan in 1991 from sick horses involved in an equine influenza outbreak (Adeyefa and McCauley, 1994). We also compared this sequence with those of other H3N8 equine influenza viruses isolated and contemporarily circulating at the same time to establish the genetic differences in relation to the antigenic drift observed in equine H3N8 subtype viruses.

MATERIALS AND METHODS

Virus growth
Ib4 was grown in 10 day-old embryonated chicken eggs at 35°C for 48-72hr, harvested, clarified and tested for haemagglutination of 1% chicken red blood cells. Chicken embryo fibroblast monolayer cells were infected with harvested infectious allantoic fluid at a multiplicity of infection (m.o.i.) of >10 pfu. Infected cell RNA was extracted 8 hr post- infection as previously described (Adeyefa *et al*., 1994) using the phenol-chloroform-iso-amyl alcohol extraction method.

The RNA was stored at -20°C in aliquots of 1µg/µl until required.

RNA sequencing

The complete nucleotide sequence of the HA gene segment of Ib4 was determined with the dideoxynucleotide (d/ddNTP) chain termination method of Sanger etal. (1977) as previously described (Adeyefa *et al*.,1994, 1996, Adeyefa and McCauley, 1997a or b?) using the silver staining sequencing kit (Promega, Southampton, UK). The nucleotide and deduced amino acid sequences were compared with those in the database at the European Bioinformatics Institute. Hinxton, UK (World Wide Wed 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 as earlier reported (Binns *et al* 1993, Adeyefa *et al*., 1994, 1996) . The Ras Mol 2.7 programme was used to map amino acid changes in the HA molecule of Ib4, Suffolk89 and contemporary viruses to antigenic sites and other functional domains on one HA monomer.

RESULTS

Figure 1 shows the complete nucleotide sequence of Ib4 HA gene segment. Compared with those of contemporary viruses, five nucleotide changes were observed at positions 45 (A→G), 478 (G→C), 562 (T→G), 805 (C→T) in the HA1 and 1188 (G→A) in the HA2 which resulted in amino acid changes (any implications of these changes on the biological functions of the HA protein?). Figure 2 shows the equine H3 HA monomer of 1989/1993 cluster (the label on Figure 2 reads 1989/91 cluster) viruses indicating amino acid changes in Ib4 compared with Suffolk89 and other H3N8 viruses isolated between 1989 and 1993 (A/eq/Suffolk/89, A/eq/Ibadan/4/91, A/eq/Ibadan/6/91, A/eq/Ibadan/6/91(repetition), A/eq/Ibadan/9/91, A/eq/Taby/91, A/eq/Arundel/12369/91 and A/eq/La Plata/1/93) that formed the 1989/1993 cluster (Fig.2)), Adeyefa and McCauley, 1997b). Yellow and orange colour shows amino acid changes in the cluster viruses while red colour (thin arrows) shows changes unique to Ib4 at antigenic sites A to D and in the HA2 peptide. HA1 is light blue (diamond arrow) and HA2 is dark blue (thick arrows). The nucleotide and amino acid changes peculiar to A/eq/Ibadan/6/91 and A/eq/Ibadan/9/91 have been previously reported (Adeyefa *et al*., 1996, Adeyefa *et al*., 1997a).

Amino acid changes occurred at residues 6 (I→V) within the signal sequence (McCauley *et al*., 1979, McCauley and Mahy, 1983, McCauley, 1987), 135 (R→T), 178 (I→T) and 244 (T→M) in HA1 and residue 43 at the amino terminal end of HA2 fusion peptide. The change in HA2 domain is unique to Ib4. These amino acid changes map to functional domains on the HA molecule: the signal sequence and antigenic sites A, C and D respectively.

Compared with the prototype European strain in the 1989/1993 (1989/1991?) cluster, Suffolk89, amino acid changes occurred at 4 residues: 6, 135 and 244 in HA1 and 43 in HA2, two of which (135 and 244) map to antigenic sites A and C respectively in HA1 described by Wiley and Skehel (1978), one map to signal sequence in HA1 while the fourth residue is in HA2. One of the changes (residue 135 R→T) led to the introduction of a new site for N-linked glycosylation at residue 133 close by (Adeyefa *et al*., 1996).

DISCUSSION

The amino acid changes observed in Ib4 are similar to those reported for A/eq/Ibadan/6/91 (Adeyefa *et al*., 1996). The change at residue 135 introduces a new N-linked glycosylation site at residue 133 closeby. There is no direct evidence for its usebesides increased infectivity observed in the virus isolated from sick horses involved in the outbreak in 1991 and in animals experimentally infected with the virus (Adeyefa and McCauley, 1994, Adeyefa *et al*., 1996, Adeyefa *et al*., 1997b) but the mobility of the HA in SDS-PAGE appeared distinct from that of viruses that do not have this site (Adeyefa *et al*., 2000). This is similar to the acquisition by an epidemic strain of human influenza A virus (A/England/878/69 H3N2) of a new glycosylation site at position 63 which also affected its antigenicity and recognition by monoclonal antibodies (Skehel *et al*., 1984). Amino acid changes were seen at residues 6, 135, 178, 244 in HA1 and 43 in HA2. These sites have not been described as those subject to host cell selection in human H3 viruses (reviewed by Robertson,1993). There could be two alternative explanations for micro-heterogeneity among closely related viruses isolated contemporarily: simple antigenic drift or heterogeneity as a property imposed by passage of virus in tissue culture (Ilobi *et al*., 1994).

Comparison of amino acid sequences of Ib4 and the other cluster viruses revealed the conservation among the HAs of two amino acids: the proline proximal to the C-terminus of HA1 and the terminal glycine of HA2. Also revealed is the conserved arginine residue at the C-terminus of HA1 which is the cleavage site conserved in all influenza A viruses examined till date except H14 strains (Kawaoka *et al*., 1990).
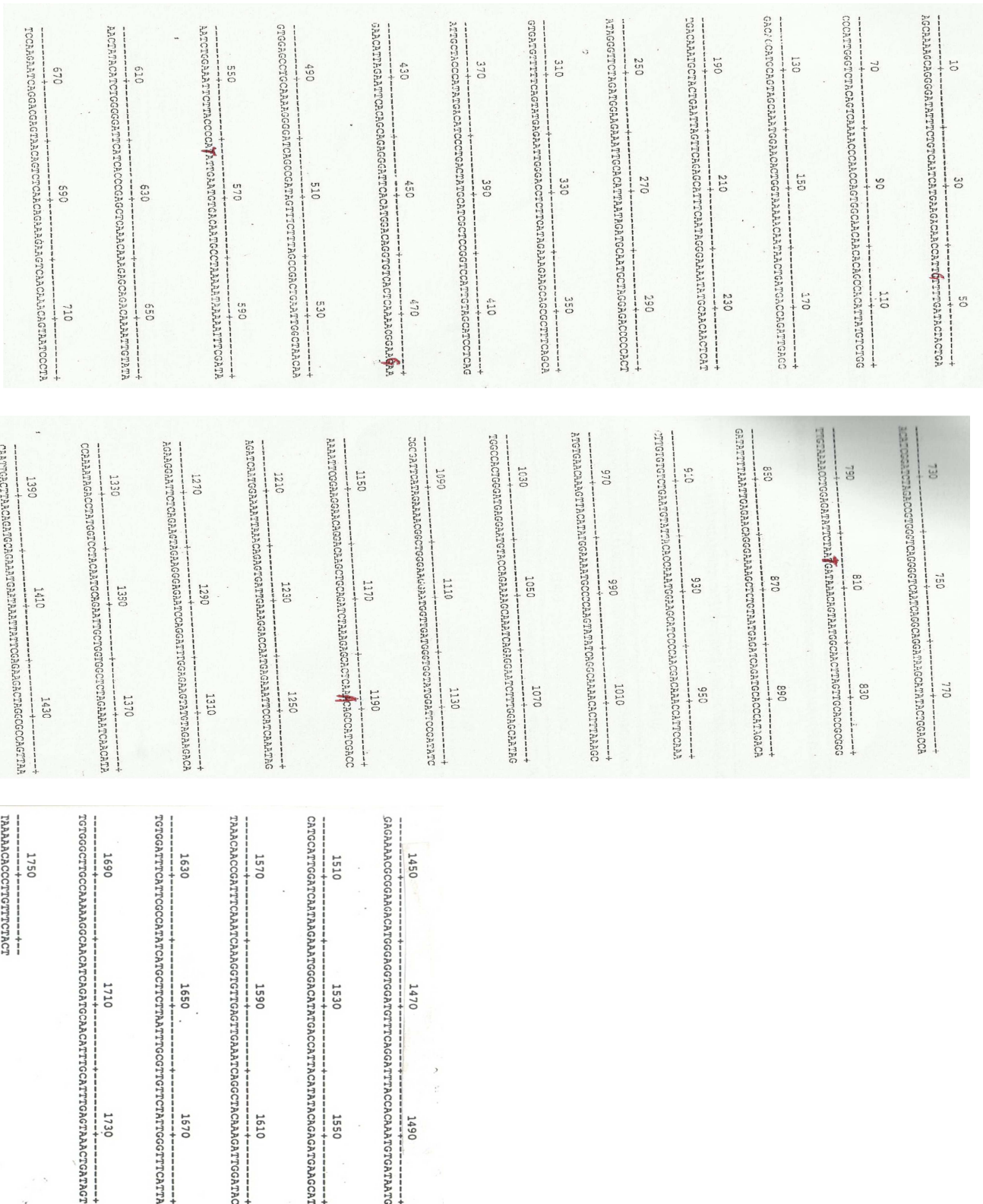
Figure 1
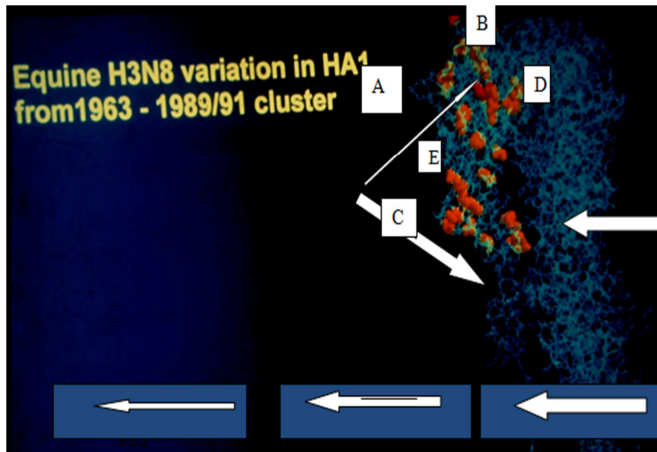Complete nucleotide sequence of A/equine/Ibadan/4/91 (H3N8)- Ib4

Figure 2
Equine H3 HA monomer showing amino acid changes in Ib4 and 1989/91

This arginine residue is absolutely required for cleavage of the HA by endogenous proteases and it is very important for abolition of steric hindrance near the cleavage site. The amino acid changes observed in Ib4 compared with the other viruses indicate antigenic drift among H3N8 equine influenza viruses (Kawaoka *et al*., 1989, Chambers, 1994, Adeyefa and McCauley, 1997b, Adeyefa *et al*.,2000). These changes occurred in functional domains of the HA, the signal sequence and the antigenic sites, which may have implications for increased virulence as earlier observed (Adeyefa and McCauley, 1994, Adeyefa *et al*., 1997b).

The method of rapid RNA sequencing in ELISA plates used in this study provides a sensitive and specific means of diagnosing and characterizing influenzavirus genes and identifying the origin of the gene segments. It does not require the use of restrictionenzyme approach to determine the origin of virus genes as reported by Offringa *et al*. (2000). The method has an advantage over the multiplex RT-PCR or the one-step RT-PCR and cycle sequencing earlier described (Adeyefa *et al*., 1994, 1996, Monne *et al*., 2008, Owoade *et al*., 2008, Cattoli *et al*., 2009) and the method of direct RNA sequencing from clinical samples earlier reported (Adeyefa and McCauley, 1997a) in that it obviates the risk of amplifying contaminating nucleic acids (RNA or DNA) to which PCR is prone or sequencing cellular or other viral nucleic acids which may arise in clinical samples in mixed infections other than those of infecting influenza virus(es). The method can also be used to establish evolutionary relationship among viruses from different hosts in diverse geographic areas of the world by partial sequence analysis of conserved regions of the viruses particularly the recently emerged pandemic (H1N1)

2009 viruses. It avoids the higher error frequency of *Taq* polymerase enzyme generally used in PCR compared to the error frequency of RNA-dependent RNA polymerase of influenza virus during transcription and replication in natural infections. It is also the method of choice where state-of-the-art facilities and funds are limiting factors.

Although mutational selection may occur during passage of virus in eggs to high titers for virus purification, in our experience, passaging virus two or three times to yield a m.o.i. of >10 pfu does not yield sub-genomic RNA or defective interfering particles that would distort the correct RNA sequence of infecting virus. Similarly, selection of variant strains imposed by several passages in tissue culture rather than in eggs (Robertson, 1993, Ilobi *et al*., 1994) and the amino acid changes observed in the present study have not been previously associated with egg adaptation of the infecting virus. Thus, the nucleotide sequence generated and the deduced amino acid sequence are adjudged to be those present in the original virus. Ambiguities that sometimes accompany cycle sequencing of PCR products were also not observed in this study.

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