MOLECULAR AND ANTIGENIC CHARACTERISATION OF INFLUENZA VIRUSES ISOLATED FROM HORSES IN NIGERIA

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ABSTRACT

A nationwide equine influenza virus surveillance was carried out between January 1989 and January 1995 during which nasopharyngeal swabs, tracheal washes and respiratory tract tissue homogenates were obtained from symptomatic and asymptomatic equine species in various parts of Nigeria for virus isolation and characterisation. Three equine influenza viruses were independently isolated in 10-11 day old embryonated hen eggs and Madin Darby canine kidney monolayer cells with TPCK-trypsin incorporated into the culture medium. The virus isolates were characterized antigenically, immunogenically and genetically which made them the first equine influenza viruses isolated and characterized in tropical Africa.

Antigenic analyses with mono-specific antisera raised against a number of respiratory disease viruses including Paramyxoviruses, Adenoviruses, Herpes viruses, Alphavirus, Rhinoviruses, Equine arteritis viruses, African horse sickness viruses and Influenza viruses showed that the three virus isolates were influenza A viruses while haemagglutination and neuraminidase inhibition assays using influenza virus specific Mabs and polyclonal antisera showed the three viruses to be antigenically H3N8, members of equine-2 subtype of influenza A viruses. The three isolates were respectively named A/Equine/Ibadan/4/91, A/Equine/Ibadan/6/91 and A/Equine/Ibadan/9/91 in accordance with the recommendations of the World Health Organisation and the International Committee on Virus Taxonomy. Their antigenic cross-reactivity with panels of Mabs and polyclonal antisera indicated that the three viruses were antigenically divergent although they were all H3N8 viruses and were representatives of a homogenous population. They were reactive with Mabs and

antisera directed against H3 equine viruses isolated in 1963, 1976, 1979, 1981 and 1986. This broad reactivity suggested that these isolates were similar to but not still different from those isolated in Europe and USA between 1963 and 1987.

Virus protein synthesis and cleavability of the HA polypeptide in tissue culture were investigated by pulse-chase experiments. The results showed some heterogeneity in the non-glycosylated polypeptides particularly those of the ribonucleoprotein (RNP) complex while the HA glycoproteins of the three viruses were not cleaved in any of the cell types used in contrast to equine H7 and pathogenic avian H5 and H7 HAs. The molecular weights of the polypeptides were within the range previously determined for influenza A viruses.

The heterogeneity of the RNP complex and antigenic divergence of the viruses' HAs were confirmed by sequence analysis carried out in molecular studies.

Virus infectivity was investigated by plaque assay using chicken embryo fibroblast (CEF) monolayer cells and virus titration in embryonated hen eggs. The results showed that the viruses were infectious with plaguing efficiency being comparable to efficiency of virus infectivity in embryonated hen eggs. Infectivity, antigenicity and immunogenicity of the three viruses were confirmed *in-vitro* using peripheral blood lymphocytes in lymphoproliferation assays and *in-vivo* in an equine model in which the viruses induced haemagglutination inhibiting and protective neutralising antibodies following experimental infections. The proliferating cells were also characterised and the immunoglobulin isotypes produced were determined.

Molecular characterisation and genetic analyses of the three viruses were accomplished to determine the origin of the genes encoding the virus non-glycosylated polypeptides as well as those of the surface HA glycoprotein. Reverse transcription (RT) results showed the eight RNA segments of the three isolates and confirmed them as influenza A virus RNAs. No subgenomic RNAs or defective interfering particles were observed in the RNA transcripts. Nucleotide sequence analyses were carried out using three sequencing strategies of cDNA, vRNA and plasmid DNA sequencing with the dideoxy chain termination procedure.

Partial nucleotide sequences were obtained from cDNAs, vRNAs and plasmid DNA clones of RNA segments 1, 2, 3, 5, 7 and 8 with the method of multiplex RT/PCR and cycle sequencing using radiolabelled segment specific oigonucleotides of 18, 24 or 25 mers. Complete nucleotide sequences of segment 4 (HA genes) were also determined on the same PCR products (cDNA) and vRNA using end-labelled oligonucleotides of both plus and minus sense. The partial nucleotide sequence data were analysed using a programme for "best-local-homology- rapid-search" on a digital array processor while the complete HA nucleotide and deduced amino acid sequence analyses were carried out using the University of Wisconsin Genetics Computer Group (GCG) package of programmes. Phytogenetic analysis was done with the distances, neighbour joining and DNAPARS of the PHYLIP package. Analyses of the viruses' gene sequences confirmed that their genomes were similar to each other and to those of other H3N8 influenza viruses isolated from equines and also revealed the origin, evolution and genetic relatedness of the genes.

Comparison of the partial cDNA sequences with virus DNA sequences in the database (EMBL sequence library) showed that for segments 1, 2, 5, and 7, the closest related sequences were from equine H3 viruses isolated in 1986 in USA (Tennesse/5/86 for segments 2, 5 and 7, Kentucky/2/86 for segments 1 and 5. Segment 5 was equally related to both viruses). The nucleotide sequence for segment 3 was most closely related to an equine-1 virus isolated in U.K. in 1973 (London/1416/73, H7N7) probably due to genetic exchange while segment 8 sequence was most closely related to an equine H3N8 virus isolated in U.K. in 1976 (Newmarket/76). The complete nucleotide and deduced amino acid sequences as well as phytogenetic analysis of the HA genes (RNA segment 4) showed a closer relationship albeit with nucleotide and amino acid substitutions between the three Ibadan viruses and those that were isolated in Europe in 1989 and 1991, the prototypic European strain, Suffolk/89 and Arundel/12369/91 isolated in U.K., Taby/91 isolated in Sweden, Hong Kong/92 isolated in the Far East in 1992 and Laplata/1/93 isolated in South America. These findings group the Ibadan viruses with those predominating and contemporarily causing disease in the Western Hemisphere rather than with viruses previously isolated from the north and south of African continent.

Variation was observed in the nucleotide sequences of the Ibadan viruses HA genes. Some of the base changes resulted in amino acid changes which mapped to antigenic sites or within signal sequence in the HA1 domain as a result of a process of antigenic or genetic drift. The Ibadan viruses also showed some variation from the prototypic European virus (Suffolk/89) and these base changes also resulted in amino acid changes resulting in antigenic drift. Phylogenetic analysis showed the evolutionary

lineages in equine H3 viruses isolated since 1963 along two paths one of which included the Ibadan viruses as well as viruses isolated in Europe between 1989 and 1991 and the Far East in 1992 and S. America in 1993 which form the 1989/93 cluster while the other lineage included viruses isolated in South America in 1987 and 1988 (Brazil/87, Laplata/88) and in the Far East in 1971 (Tokyo/3/71) all of which are very close to the original prototype equine-2 virus (Miami/63). These results demonstrate a faster evolutionary rate for recent equine H3 HA genes away from the original prototype virus.

Overall, the results of these studies have (i) confirmed the occurrence of equine-2 H3N8 influenza viruses of distinct lineages in Nigerian equine populations in a tropical environment (ii) indicated antigenic drift among equine H3N8 viruses as earlier reported and confirmed that drift strains can co-circulate in equine populations, (iii) showed the origin, evolution and genetic relatedness of the viruses genes as well as their biological characteristics, (iv) provided the hitherto unavailable information on the status of equine influenza virus in this part of the world and (v) served to re-emphasise the potential of influenza virus for rapid global spread and the need for better control strategies.

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CERTIFICATION

THIS IS TO CERTIFY THAT THIS WORK WAS CARRIED OUT BY

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DEDICATION

This work is dedicated to the Glory of Lord God Almighty as reflected in my family - my wife and children - Oluwaseyi, Oluwagbemisola and Oluwagbade. It is also dedicated to my parents, Eddy and Felicia, to Adiaha Ecaete for a dream fulfilled, to the memory of my late brother, Idowu and to all those scientists all over the world toiling day and night in various endeavours for the well-being of mankind and other creatures great and small.

LIST OF ABBREVIATIONS

AMV	--	avian myeloblastosis virus
APS	--	ammonium persulphate
ATP	--	adenosine triphosphate
Bisacrylamide	--	N N-methylenebisacrylamide
BSA	--	bovine serum albumin
bp	--	base pair
CEF	--	chicken embryo fibroblast
cRNA	--	complementary unpolyadenylated ribonucleic acid
dATP	--	deoxyadenosine triphosphate
dNTPs	--	deoxynucleotide triphosphate
ddNTPs	--	dideoxynucleotide triphosphate
DNA	--	deoxyribonucleic acid
cDNA	--	complementary deoxyribonucleic acid
DDT	--	dithiothreitol
DMEM	--	Dulbeceo modified Eagle's medium
EDTA	--	ethylenediamine tetra-acetic acid
ELISA	--	enzyme linked immunosorbent assay
FPV	--	fowl plague virus
hfu	--	haemagglutonin forming unit
HA	--	haemagglutonin protein, haemagglutination test
HAI	--	haemagglutonin inhibition test

FT9	--	A/Eq/Fontaineblue/79
Hepes	--	N-2-hydroxyethylpiperazine-N-2 ethanosulphonic acid
IB4, IB6, IB9	--	A/Eq/Ibadan/4/91, A/Eq/Ibadan/6/91, A/Eq/Ibadan/9/91
Kb	--	kilobase
Mabs	--	Monoclonal antibodies
M	--	matrix protein
M2	--	second matrix protein (from spliced mRNA) of segment 7
M63	--	A/Eq/Miami/63
MmLK	--	Moloney murine leukaemia virus
mRNA	--	messenger ribonucleic acid
NA	--	neuraminidase protein
NAI	--	neuraminidase inhibition test
NP	--	nucleoprotein
NS1	--	non-structural I protein
NS2	--	non-structural 2 protein
nt	--	nucleotide
PA	--	polymerase acidic protein
PB1	--	polymerase basic protein 1
PB2	--	polymerase basic protein 2
PBS	--	phosphate buffered saline

PBL	--	Peripheral blood lymphocytes
PCR	--	polymerase chain reaction
pfu	--	plaque forming unit
Rbc	--	red blood cells
RNA	--	ribonucleic acid
RNAse	--	ribonuclease
RNP	--	ribonucleoprotein
rpm	--	revolutions per minute
RT	--	reverse transcription
SDS	--	sodium dodecyl sulphate
SI	--	stimulation index
TBE	--	Tris-borate EDTA buffer
TE	--	Tris EDTA buffer
TER	--	Tris EDTA ribonuclease buffer
TEMED	--	NNN N-tetramethylethylenediamine
TBF	--	transformation buffer
tRNA	--	transfer ribonucleic acid
ts	--	temperature sensitive
μl, μg	--	microliter, microgram
mg	--	milligram
UV	--	ultraviolet
vRNA	--	virion ribonucleic acid
X-gal	--	5-bromo-4-chloro-3-indoxyl-B-D-galactosidase

TABLE OF CONTENTS

CHAPTER 2 ISOLATION, IDENTIFICATION, ANTIGENIC AND POLYPEPTIDE ANALYSES OF EQUINE INFLUENZA VIRUSES

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

INTRODUCTION

The influenza virus belongs to the **Family** *Orthomyxoviridae*, **Genus** *Influenza*. It is a segmented negative sense, single stranded RNA virus and the genome is complementary to translatable positive sense mRNA. Influenza viruses can be divided into three immunologically distinct serotypes A, B and C based on their antibody reactions to the virus core proteins, the polymerase (P), matrix (M) and nucleoprotein (NP) all of which are serotype specific (Akkina, 1990). There are 8 segments, each of about 0.2-1 x 10^6 kilodalton (KD) molecular weight (M_r) and a total M_r of 5 x 10^6 KD for influenza A and B viruses (Matthews, 1979).

Influenza A viruses undergo significant antigenic variation and a high level of genetic recombination giving rise to antigenic drift and genetic shift (Murphy and Webster, 1990) due to the genome's division into 8 separate single strands of RNA (McGeotch *et al*, 1976) and are thus very unique among the respiratory disease viruses. Influenza viruses infect a wide range of mammalian and avian hosts in a wide spectrum of geographical locations (Gorman *et al.*, 1990a; Yasuda *et al.*, 1991). The viruses also cause a disease of high morbidity and mortality with great economic losses in terms of human resources and valuable animals and birds.

The history of influenza dates back to long before 412 B.C. since when epidemics/pandemics of varying degree of severity have occurred frequently in mammals and birds with occasional disappearances of circulating viruses and emergence of new ones (Noble, 1982).

In influenza A viruses 14 haemagglutinin (HA) and 9 neuraminidase (NA) subtypes are known, none of which is serologically cross-reactive (Kawaoka *et al.*, 1990). The H1N1, H2N2 and H3N2 subtypes infect humans, classical H1N1, avian-like H1N1 and human-like H3N2 infect swine, H7N7 and H3N8 infect equines (Nicholson, 1994) while all the 14 HA and the 9 NA subtypes occur in oirds of which only the H5 and H7 HA subtypes are highly pathogenic in poultry (Bean *et al.*, 1985; Webster *et al.*, 1986; Alexander *et al.*, 1993). Influenza viruses also infect primates, mink, seals and whales (Hinshaw *et al.*, 1984, 1986). The evolutionary patterns among influenza A viruses indicate that separate host-specific virus gene pools exist and inter-species transmission, combined with separation of host species contribute significantly to evolutionary divergence of these viruses (Webster *et al.*, 1992).

The terminal 13 nucleotides at the 5′ end and the 12 nucleotides at the 3′ end of influenza A viruses are conserved (Skehel and Hay, 1978; Robertson, 1979) and oligonucleotides complementary to these terminal nucleotides are frequently used to prime cDNA synthesis of either the negative or positive sense virus RNAs. The virus specific messenger RNA (mRNA) consists of molecules complementary to the genomic RNAs. In virus infected cells, 2 types of complementary copies of input genomic RNAs are made: a full length copy (cRNA) which serves as a template for genome replication and an incomplete copy (mRNA) that serves as a messenger and is translated into proteins. At the 5' end of each segment, the nucleotide sequence 5′-AGUAGAAACAAGG occurs followed by a triplex unique to each RNA segment and then a tract of oligo U which is essential

for mRNA synthesis (Luo *et al.*, 1991). At the 3′ end the sequence 3′HO-UCGUUUUGGUCC is found which is conserved in most RNA segments but found as 3′HO-UCGCUUUCGUCC in segments 1-3 and 7 of human influenza A virus strains. Apart from these no homologies exist between the segments. Similarly varying degrees of homology exist between nucleotide sequences of individual segments among the different strains. However, the 3′ terminal 15 nucleotides contain influenza virus promoter (Yamanaka *et al.*, 1991) which is the conserved nucleotide sequence of transcription start region. The termination sequences are also conserved.

Influenza virus transcription occurs in the nucleus of virus infected cells, it requires special cooperation of virus and cell activities and it is mediated by a virus-coded RNA-dependent RNA polymerase with similar function to that of RNA transcriptases of other negative strand viruses. As influenza virus transcriptase is unable to initiate mRNA synthesis or modify the 5′ termini of mRNA molecules by capping and methylation, transcription is primed by capped and methylated 5′ termini supplied by the action of cellular mRNA producing RNA polymerase II (Mayh *et al.*, 1972). Advantage is thus taken of this knowledge to initiate the reverse transcription of influenza RNA segments *in vitro* using a reverse transcriptase of other viruses (avian myeloblastosis virus, AMV or Moloney murine leukaemia virus, (MmLV) followed by synthesis of a second strand using a DNA polymerase (Taq or TSP) to generate a cDNA from which the virus gene sequences can be determined. The dideoxy chain termination sequencing procedure of Sangers *et al.* (1977) is often employed to determine virus gene nucleotide sequences. The

procedure capitalises on the ability of DNA polymerase to faithfully synthesise a complementary copy of a single-stranded DNA template and its ability to use 2′3′-dideoxynucleotide triphosphates as substrates. DNA synthesis is carried out in the presence of the four deoxynucleotide triphosphates, one or more of which is labelled with ^{32}P and in four separate incubation mixes containing low concentration of each of the four dideoxynucleotide triphosphate analogues. Once the analogue is incorporated at the growing end of the DNA chain, the 3′ end lacks a hydroxyl group and is no longer a substrate for chain elongation, thus the growing DNA chain is terminated. Therefore in each reaction, there is a population of partially synthesised radioactive DNA molecules, each having a common 5′ end but each varying in length to a base-specific 3′ end. After a suitable incubation period, the DNA in the mixture is denatured, electrophoresed side by side and the radioactive bands of single stranded DNA are detected by autoradiography. The nucleotide sequences can then be read directly from the X-ray films.

Influenza A viruses are capable of replication in a new host species and of undergoing genetic reassortment resulting in generation of novel virus(es). These can lead to antigenic drift and genetic shift which invariably result in epidemics or pandemics of influenza in different host species. In the light of these, the precise origin of the virus genes is an important characteristic of the virus. Although the study of the circulation of influenza viruses in mammalian and avian populations in a locality relies on the detection of serum antibodies to these viruses, precise knowledge of the antigenic identity of viruses prevalent in that locality is of inestimable value in the interpretation of such serological findings. Isolation, antigenic and genetic analyses of the viruses by nucleotide sequencing

often yield information on the precise identity and characteristics of the viruses including their origin, evolution and genetic relatedness.

In recent times there has been a noticeable increase in acute respiratory infections in mammalian and avian populations (Adeyefa, 1992; Adeyefa and Durojaiye, 1992) most of which go undiagnosed or mis-diagnosed. Respiratory tract infections occur commonly among competition horses particularly when they congregate for equestrian events and even sales (Adeyefa and McCauley, 1994). These infections are frequently caused by respiratory disease viruses including influenza virus, herpesvirus types 1 and 4, adenovirus and rhinovirus (Powell, 1991). The two subtypes of influenza A virus that infect horses can co-circulate but the H7N7 virus has not been isolated from the horse for over 15 years now while the H3N8 virus is the most prevalent. Reports of recent outbreaks and isolation of equine-2 influenza viruses from most parts of the world except tropical Africa and Australia abound in the literature and in view of the trans-continental movement of horses for competition and breeding, the incidence of these outbreaks worldwide appear to be increasing as evidenced from reports from Western Europe (Donatelli *et al.*, 1991; Wood and Mumford 1992; Binns *et al.*, 1993), Scandinavia (Oxburg *et al.*, 1993), North and South America (Kawaoka *et al.*, 1989; Endo *et al.*, 1992; Webster and Kawaoka, 1994; Lai *et al.*, 1994), Indian sub-continent (Rana *et al.*, 1992; Gupta *et al.*, 1993), South Africa (Kawaoka and Webster, 1989), China, Japan and Hong Kong in the Far East (Guo *et al.*, 1992; a, b; Webster and Kawaoka, 1994; Lai *et al.*; Guo *et al.*, 1994; Shortridge *et al.*, 1995). Although antibodies to some human influenza virus strains have recently

been demonstrated in the sera of horses, pigs and chickens (Adeniji *et al.*, 1993), there is a dearth of information on the occurrence, clinical manifestation, virus isolation and characterisation as well as epidemiology of equine influenza in tropical Africa particularly Nigeria to my knowledge. It is thus conceivable and expedient to endeavour to carry out studies which should yield such hitherto unavailable information on the status of equine influenza virus in this region of the world.

AIMS AND OBJECTIVES OF RESEARCH PROJECT

(i) To carry out a nationwide equine influenza virus surveillance in Nigeria and isolate viruses from symptomatic and asymptomatic equine populations in various parts of the country.

(ii) To analyse the virus isolate(s) antigenically and immunogenically.

(iii) To characterise the virus isolate(s) at the molecular level by determining the nucleotide and amino acid sequences of their genes.

(iv) To determine the origin, evolution and genetic relatedness of the virus genes.

The results from these studies would provide the hitherto unavailable information on equine influenza virus in this environment and thus establish a record of genetic and epidemiological significance on which further work could be based. The knowledge of the characteristics of currently circulating viruses among Nigerian equine populations could be utilised for effective control of influenza among valuable animals by modifying or upgrading currently available vaccines or even designing new immunogens and by formulating better control strategies. This is also applicable with other respiratory disease viruses and viruses generally causing infections in different host species.

CHAPTER 1

LITERATURE REVIEW

There are three serotypes A, B and C of influenza virus and their core protein antigens are serotype specific (Akkina, 1990). Influenza A viruses are wide-spread in nature and infect a wide variety of host species including mammals and birds while influenza B and C viruses infect only human hosts although influenza C virus had been isolated from pigs in China (Guo *et al.*, 1983). Influenza viruses can undergo antigenic drift and genetic reassortment which results in the generation of a novel virus. Interspecies transmission also occurs particularly with influenza A viruses on which this review will be mostly focused. The review will also focus on the structure and function of influenza virus genome, epidemiology in various host species, the origin, evolution and genetic relatedness of influenza A virus genes, pathogenesis, clinical manifestation, immunity, diagnosis as well as treatment and control in various host species with particular emphasis on the equine hosts.

1.1 CLASSIFICATION AND NUMENCLATURE

Influenza viruses are classified among the myxoviruses which include Families *Orthomyxoviridae* and *Paramyxoviridae* based on their special affinities for mucopolysaccharides and glycoproteins particularly sialic acid-containing receptors on cell surfaces. Members of the two families share many similarities but differ greatly in their genetic behaviour, intracellular sites of antigen accumulation and sensitivity to drugs that affect nucleic acid and protein synthesis (Waterson, 1962). Influenza viruses are named

based on type and subtype of the virus, the host species from which they are isolated, place and year of isolation and the numbers of each isolate. Thus an eighth influenza A isolate, subtype H1N1 obtained in Puerto Rico from a man in 1934 is designated A/Puerto Rico/8/34 (H1N1). In animals and birds the host species is placed before the place of isolation, thus in equines for example the first isolate of subtype H7N7 influenza A virus from the horse obtained in Prague in 1956 is called A/Equine/Prague/1/56 (H7N7) and a subtype H3N8 isolated from horses in Miami in 1963 is designated A/Equine/Miami/63 (H3N8). B and C types of influenza virus are also named as the A type in man, e.g. B/Lee/40 and C/Ann Arbor/50.

1.2 INFLUENZA VIRUS STRUCTURE AND COMPOSITION

1.2.1 Virus Particle

Electron microscopic studies of influenza viruses have revealed the virus particle to be about 80-120nm in diameter, mostly spherical but sometimes elongated and filamentous as in newly isolated strains (Hoyle, 1968; Nermut and Frank, 1971; Erickson, 1977). The mass of influenza A and B viruses is estimated to be 174 x 10^6 daltons. A lipid bilayer about 40-50 Å thick constitutes 25-28% of the virion mass while the surface glycoproteins of 400-500 spikes comprise 40-46% of the total mass of the virus particle (Ruigrok *et al.*, 1984).

1.2.2 The Virus Genome

The genomes of influenza A and B viruses comprise 10 genes shared among 8 RNA segments. Segments 4 and 6 are devoted to the envelope glycoproteins while in influenza C virus only a single glycoprotein is encoded by segment 4, thus having one less RNA segment and specifying 8-9 proteins. Influenza A virus genome consists of 8 single-stranded negative sense RNA molecules which are present in the virus particle in equimolar amounts. The two smallest ones give rise to two mRNAs, one collinear and the other derived by splicing (Scholtissek *et al.*, 1993). The genomic RNA segments are associated in the mature virion with a basic arginine-rich protein comprising 498 amino acids with a M_r of 56,000 or 56K called the nucleoprotein (NP). There are about 1,000 NP molecules per virion (Inglis *et al.*, 1976). The NP along with the three polymerase (P) polypeptides and RNA forms the virus core which is the nucleocapsid with RNA polymerase activity (Scholtissek *et al.*, 1993; Figs. 1a & 1b). Each influenza virus gene resides in a separate piece of RNA and is thus individually encapsidated as a ribonucleoprotein (RNP). The RNP complexes form large left handed helices which are sometimes more extended that they are distinct from the narrow helices of the nucleocapsid segments (Heggeness *et al.*, 1985) which are exposed to ribonuclease. The three types of influenza virus differ on the basis of their RNP antigenicity.

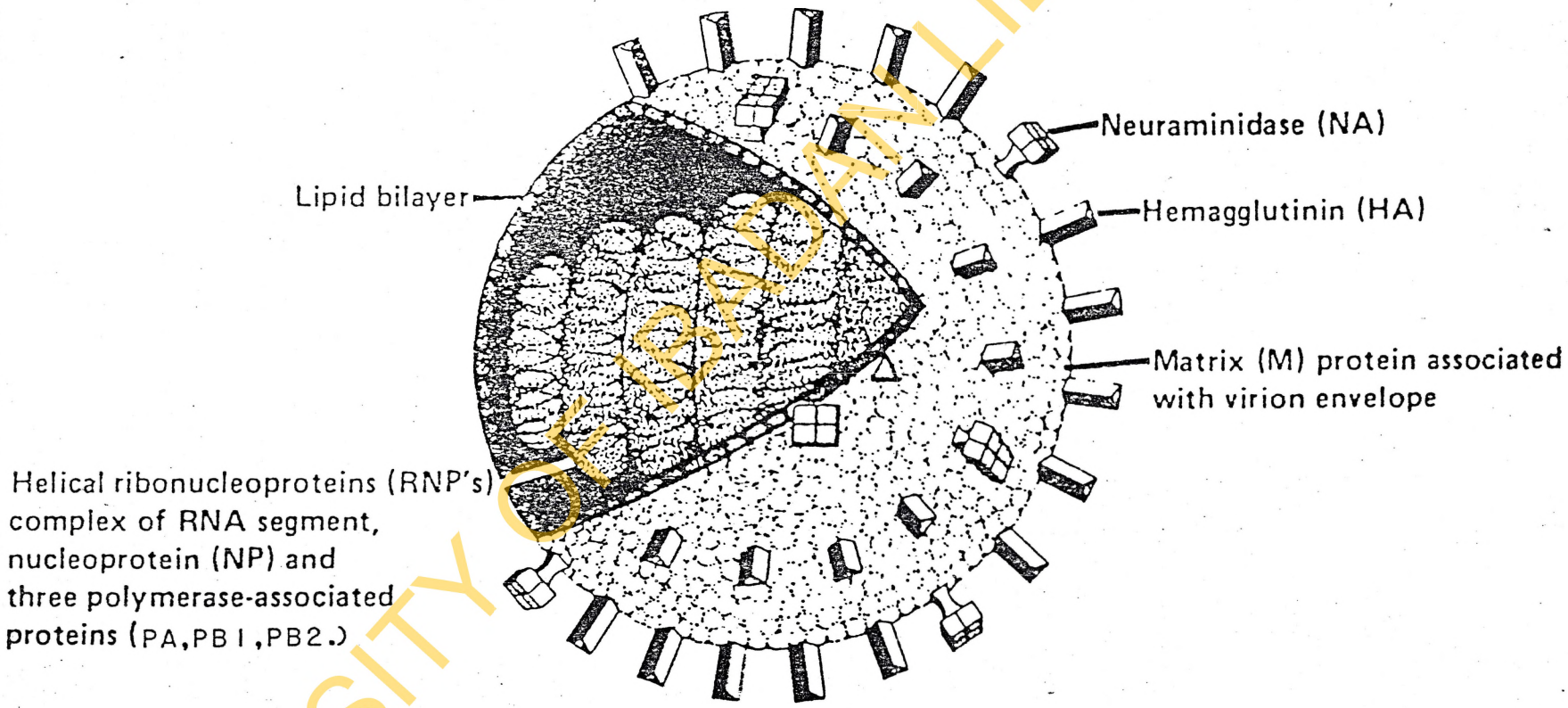


Fig. 1a. Structure of influenza virus particle. Adapted from Wilson et al (1981).

Fig. 1a. Influenza virus particle.

10

Fig. 1b. Structure and composition.

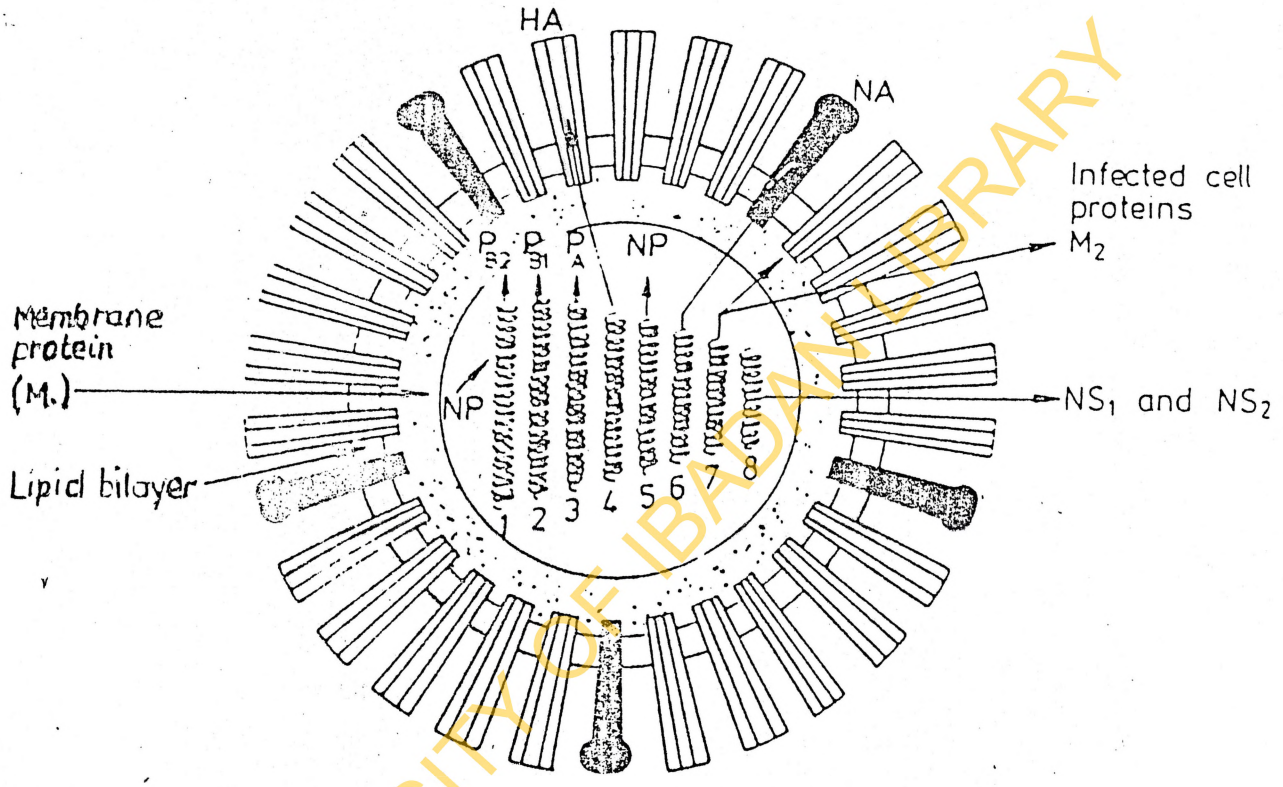


Fig. 1b: Model of influenza virus showing protein and RNA composition. Adapted from Wilson et al., (1981)

The RNP is surrounded in the virion by a lipid bilayer underlined by a shell of matrix protein (M1) which is hydrophobic and consists of 252 amino acids (Figs. 1a & 1b). The M_r is about 28K and there are about 2,500 molecules of M1 protein per virion (Inglis *et al.*, 1976; McCauley *et al.*, 1982). The M1 protein lies beneath the viral envelope, it is soluble in organic solvents and is phosphorylated close to the major stretch of neutral and hydrophobic amino acids, although the significance of this phosphorylation is yet to be clarified (Gregoriades *et al.*, 1990). M2 is also a membrane associated protein detectable in virus particles at lower levels than those in the cell and it is not glycosylated (Zebedee *et al.*, 1985) though it has been found to possess associated palmitate and may form a tetrameric proton ion channels to control the viral internal pH (Sugrue *et al.*, 1990; Veit *et al.*, 1991). A few molecules per virus particle of the M2 protein which is synthesised on the spliced mRNA of segment 7 (M gene) span the lipid bilayer (Scholtissek *et al.*, 1993) and single amino acid changes in M2 can render influenza A viruses resistant to amantadine and rimantadine (Nicholson, 1994). Within the matrix shell and in association with the genomic RNA are the three high molecular weight polymerase (P) proteins which form an RNA dependent polymerase complex capable of transcribing complementary mRNA molecules from the genomic RNA template (Kawakami and Ishihama, 1983). PB1 and PB2 are basic and comprise 757 and 759 amino acids respectively while the third, PA is acidic and comprise 716 amino acids (Fields and Winter, 1982; Fig. 1b).

The matrix protein shell of the virus particle is surrounded by a host-cell derived lipid bilayer into which are inserted virus coded protein spike-like projections prior to budding of the virus particle (Compans *et al.*, 1970). Following glycosylation of these protein spikes by the host cell, the two antigenic surface glycoproteins are inserted into the membrane by their uncharged hydrophobic anchor regions. About 80% of these projections are haemagglutinin (HA) and the remaining 20% are neuraminidase (NA). The HA is a trimer with a M_r of about 63 - 65K. It is triangular in cross-section, measures 13.5nm from membrane to tip with a varying radius of 1.5-4nm and consists of three identical subunits each containing two polypeptide chains HA1 and HA2 (Fig. 2a) linked in each subunit by a single disulphide bond (Waterfield *et al.*, 1981). Post-translational cleavage of the nascent HA monomer either during or after viral replication removes an arginine to divide the chains into 2 disulphide-linked chains HA1 of 328 amino acids and HA2 of 221 amino acids, the cleavage being required for viral infectivity and HA-mediated fusion activity (Ward and Dopheide, 1979; 1980; Haung *et al.*, 1980). The HA comprises two structurally distinct regions; a triple-stranded coiled coil of α-helices extending from the viral membrane and a globular region of eight-stranded ß-sheet structure which is distal to the coiled coils of the fibrous stalk-like region that stabilises the trimer and serves as a stem that unites the globular region with the viral membrane at which is a five-stranded ß-sheet containing the N-terminus of HA1 and the C-terminus of HA2 (Figs. 2a & 2b). Crystallisation of sialic acid in the HA binding site

Fig. 2a. Influenza virus haemagglutonin trimer inserted into viral membrane

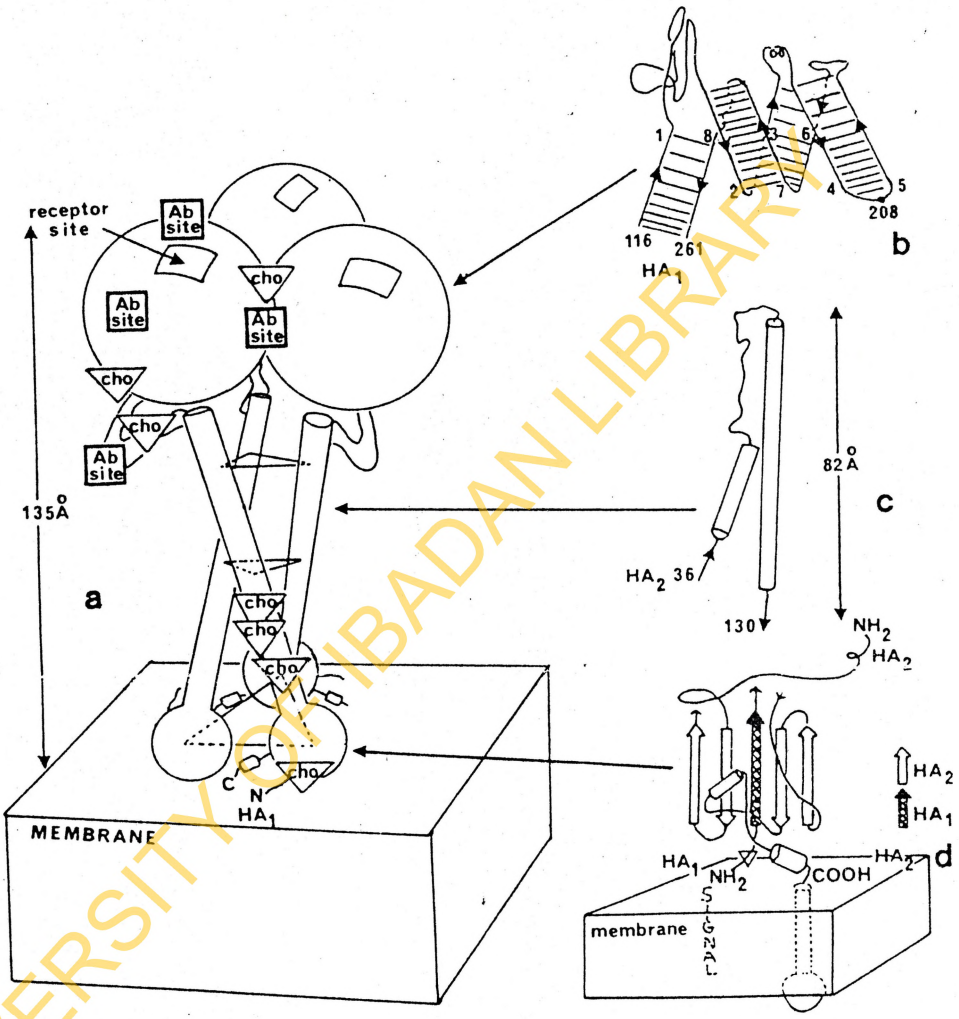


Figure 2a: Diagrammatic representation of HA trimer inserted into the viral membrane (From Wilson et al., 1981.

Fig. 2b. Influenza virus haemagglutonin (HA) and neuraminidase (NA) structure.

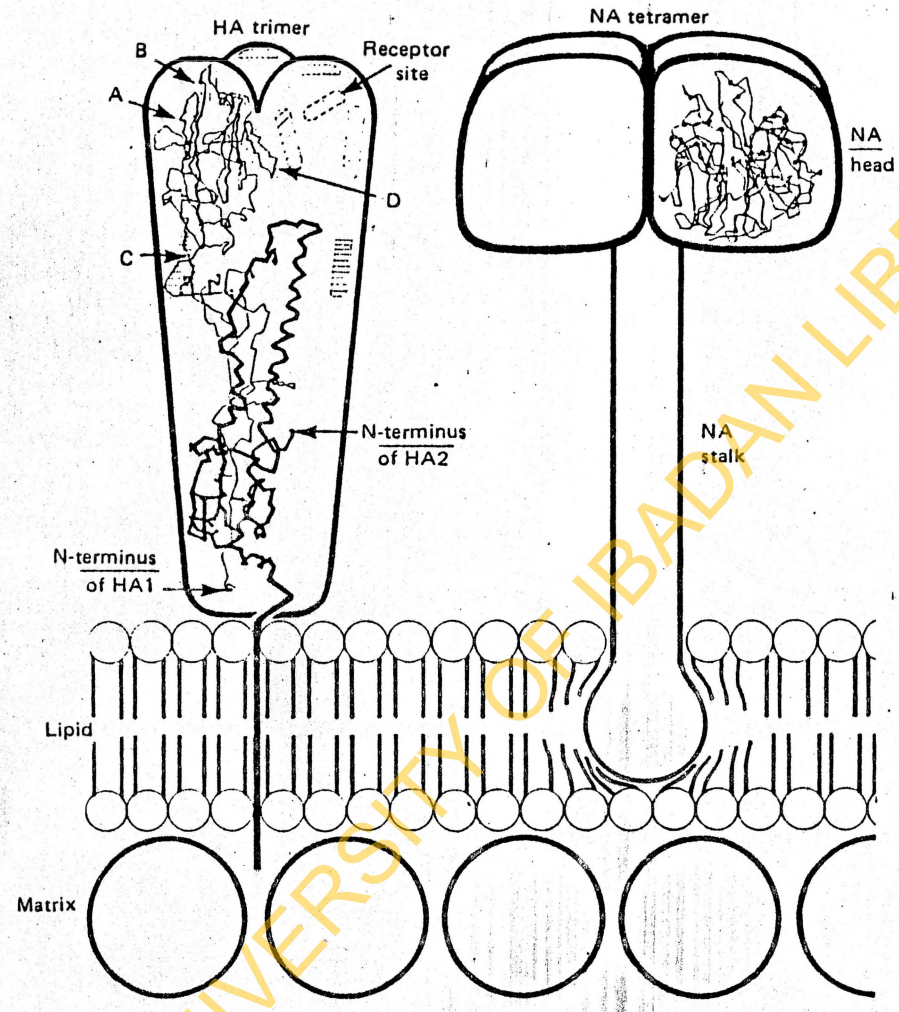


Fig. 2b Diagrammatic representation of influenza virus HA and NA structure.

The HA trimer and NA tetramer glycoproteins are inserted in the viral lipid membrane. A carbon chain representation of the polypeptide (from x-ray crystallography studies by Wilson *et al.*, 1981; Colman *et al.*, 1983) is shown for a single HA and NA subunit. HA2 polypeptide is designated in thicker line whilst HA1 is shown in thinner line. Note the position of the receptor site on HA and either 4 or 5 antigenic sites (designated A-C, depending on whether site C is subdivided into two antigenic sites). The N-terminus of HA2 has fusion activity whilst the C-terminus of HA2 penetrates through the lipid layer to come into approximate contact with the M monolayer. The epitopes on the NA are more widely distributed around the enzyme active site on the head.

Fig. 2c. Sub-unit folding of N2 neuraminidase.

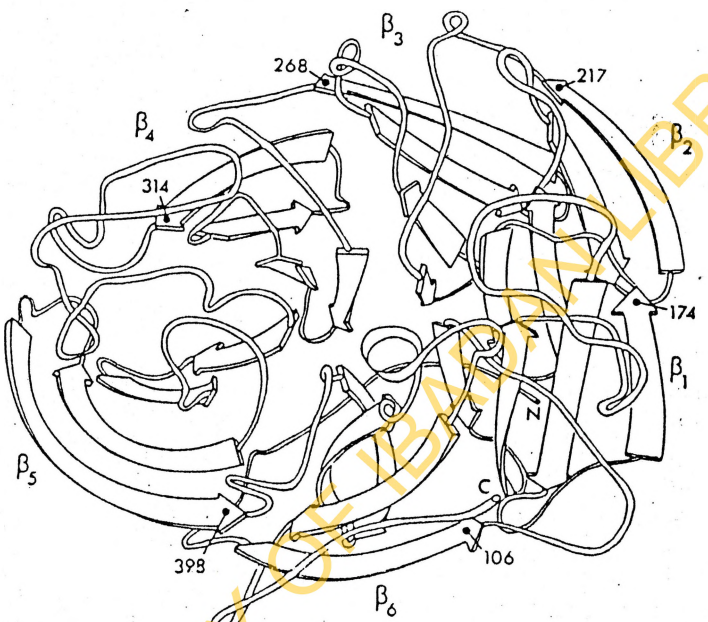


Fig. 2c Diagram of subunit folding of N2 neuraminidase. The molecular fourfold symmetry axis is indicated, and the N-terminal segment emanating from that axis is on the underneath side of the subunit. The view is down the symmetry axis from above. The C terminus is on the outside surface near a subunit interface. The six β sheets of the propeller fold are near 4 o'clock, 2 o'clock, 12 o'clock, 10 o'clock, 8 o'clock, and 6 o'clock, respectively. The N and C termini are indicated, as are residue numbers at the C-terminal end of each sheet. (From Varghese et al., 1983).

has shown that HA binds to sialic acid bearing glycoproteins on the host cell by means of a pocket on the head region which is linked by conserved amino acids (Weis *et al.*, 1988). The analyses of natural variants and single site laboratory antibody selected mutants with single amino acid changes has demonstrated clustering of changes in five sites on H3 HA believed to be antibody binding sites, all of which are on the head region and one depends on the trimeric form (Wiley *et al.*, 1987; Wiley and Skehel, 1987; Fig. 2a & 2b). Amino acid changes at these sites are responsible for antigenic drift (Nicholson, 1994). The NA on the other hand is a tetramer with an M_r of about 56K which unlike the HA is attached to the membrane by hydrophobic amino acid anchor. The assembled tetramer has a box-shaped head on a thin stalk (Fig. 2b). Each monomer is composed of 6 ß-pleated sheets arranged in a propeller formation (Fig. 2c). While the HA is about 19% carbohydrate, 46% of the NA molecule is carbohydrate side chains, one of which contributes to interactions in the tetramer (Tulip *et al.*, 1991; Varghese and Colman, 1991). The NA is the enzyme that cleaves sialic acid from the HA and cell surface and promotes virus release (Nicholson, 1994). The four catalytic sites together form a deeper receptor binding pocket. Thus the binding pocket on both HA and NA are shielded from antibodies. Seven antigenic sites around the pocket on the NA head form an almost continuous surface and steric hinderance by antibodies can block binding to the substrate (Colman *et al.*, 1983).

Apart from the structural proteins (M, HA and NA), six other proteins are encoded by the virus genome. These are PB1, PB2, PA, NP, NS1 and NS2 proteins of which NP, NS1 and NS2 could be phosphorylated. The role of NS1 has been attributed to the shut-off of host cell and viral protein synthesis while NS2 has been detected in

released virus at low levels (Wolstenholme *et al.*, 1980; Richardson and Akkina, 1991) and plays a role in promoting normal replication of genomic RNAs by preventing the replication of short-length sub-genomic RNA species (Odagiri *et al.*, 1994). Studies on hybrid expressed translation (Inglis *et al.*, 1977), recombination between temperature sensitive (ts) mutants (Almond and Barry, 1979) and recombination between different strains (Palase *et al.*, 1977) have elucidated the assignment of proteins to the genome segments that code for them. Each of the 6 largest RNA segments encodes a single polypeptide present in both the virion and infected cells. Thus segment 1 encodes PB2, segment 2, PB1, segment 3, PA, segment 4, HA, segment 5, NP, and segment 6, NA. The two smallest segments 7 and 8 each encodes two polypeptides M and M2 as well as NS1 and NS2 respectively (Table 1). While only M protein is found in the virion, the others are non-structural virus polypeptides. Each protein is translated from a single mRNA species. An additional eleventh species of polyadenylated capped RNA encoded by segment 7 has been isolated from infected cells. All the mRNA molecules are of opposite polarity to the genomic RNAs but no polypeptides are translated from the virion RNA sense (Fig. 3). Table 1 shows the complete coding assignment of influenza A subtype H7. The 5′ and 3′ terminal sequences of influenza A virus RNA segments are conserved between the segments and among the different subtypes (Skehel and Hay, 1978; Robertson, 1979) as in the types B and C viruses (Desselberger *et al.*, 1980). At the 5′ end of each segment in influenza A viruses, the 13 nucleotide sequence 5′-

Table 1. Influenza virus genome RNA segments and their Coding Assignment.

Segment	Average Molecular Weight	Length (nucleotides)	mRNA length (nucleotides)	Encoded Polypeptide	Nascent Polypeptide Length amino acids	Approx. No. Molecules per Virion	Structural Features	Function
1	85,800	2,341	2,320	PB2	759	30-60	Basic protein	Host cell RNA cap binding, component of RNA transcriptase
2	86,300-86,500	2,341	2,320	PB1	757	30-60	Basic protein	Initiation of transcription elongation component of RNA transcriptase
3	82,400-83,000	2,233	2,211	PA	716	30-60	Acidic protein	Component of RNA transcriptase
4	HA 77,000 HA1 50,000 HA2 27,000	1,778	1,757	HA	566	500	Glucoprotei n 3D structure	Surface glycoprotein, trimer, major antigenic determinant, entry of host cell.
5	NP 56,101	1,565	1,540	NP	498	1,000	Coiled filaments	Association with RNA segments to form ribonucleoprotein, elongation, component of RNA transcriptase.
6	NA 56,000 (tetramer 220,000)	1,413	1,392	NA	454	100	Glyoprotein 3D structure	Surface glycoprotein, tetramer, neuraminidase activity. Major protein component of virus, underlies lipid by layer
7	M 28,861	1,027	1,005 316 276	M1 M2	252 96 9	3,000 14-67		Spliced nRNA, present in virion, possible proton channel. M2 involved in antiviral activities.
8	NS	890	868 395	NS1 NS2	230 121	120		Non-structural protein, spliced mRNA, NS1 involved with post-translational processing of late proteins

Fig. 3. Schematic diagram of influenza virus vRNA, mRNA with poly A tail (mRNA poly A+) and unpolyadenylated A(-) cRNA with the conserved nucleotides at the ends of each RNA segment. Adapted from Lamb and Choppin (1983).

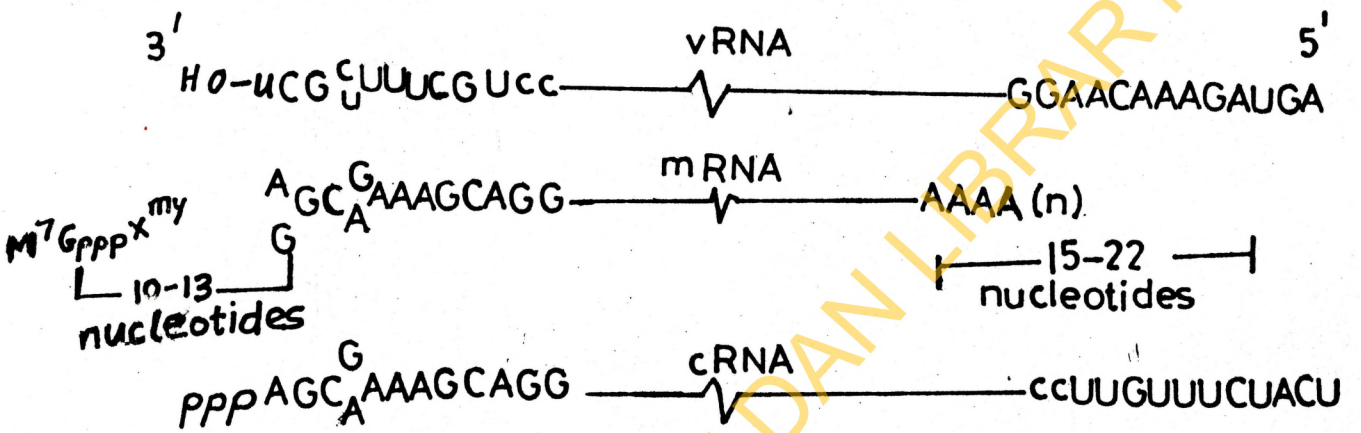


Fig.3. Schematic diagram of influenza virus vRNA, mRNA with poly A tail (mRNA poly A+) and unpolyadenylated A(-) cRNA with the conserved nucleotides at the ends of each RNA segment. Adapted from Lamb and Chopin (1983)

AGUAGAAACAAGG occurs, followed by a triplet unique to each RNA segment and then a tract of oligo U which is essential for mRNA synthesis (Luo *et al.*, 1991). At the 3′ end, a 12-nucleotide sequence of 3′HO-UCGUUUUCGUCC is found, which is conserved in most RNA segments but found as 3′HO-UCGCUUUCGUCC in segments 1-3, and in segment 7 of human influenza strains (Allen *et al.*, 1980; Winter and Fields, 1980). Apart from these no homologies exist between the segments even for the two P genes encoding PB1 and PB2 of identical nucleotide length of 2341 nucleotides. Similarly varying degree of homology exists between nucleotide sequences of individual segments between different strains. However, the 3′ terminal 15 nucleotides contain the influenza sense promoter (Parvin *et al.*, 1989, Yamanaka *et al.*, 1991). In the virus infected cells, two types of complementary copies of the input genome are made (Fig. 3); a full length copy (cRNA) which serves as a template for genome replication and an incomplete copy (mRNA) that functions as a messenger and is translated into proteins. It cannot function as a template because it is adenylated from the oligo-U tract 17-22 bases from the 5′ end of the vRNA (Hay *et al.*, 1980).

1.3 REPLICATION, TRANSCRIPTION AND TRANSLATION

A number of virus-host systems have been used to study the mechanism of replication of influenza viruses. Studies in embryonated hen eggs, chicken embryo fibroblasts (CEF) and Madin-Darby bovine and canine kidney cells (MDBK, MDCK) have yielded substantial knowledge of the replication mechanisms and control in influenza viruses (Hay *et al.*, 1976, 1980, 1982).

1.3.1 Adsorption

Infection is initiated by attachment of the HA spikes of the virus to neuraminic (sialic) acid-containing receptors on the cells. This adsorption step, though necessary for infection to evolve, does not necessarily result in infection. However, when infection occurs following adsorption a virus particle may enter the host cell by any of several basic mechanisms including direct penetration, endocytosis, fusion or a combination of these (reviewed by Dimmock, 1982). Only one virus particle is required to infect a cell for plaque formation thus displaying one hit kinetics (Sugiura and Kilbourne, 1965). First, the virus particle binds to the receptor on the host cell. The receptor for influenza viruses (sialic acid) is bound in a cleft on the large globular head region of the HA (Weis *et al.*, 1988). The binding affinity for a particular receptor depends on the species from which the virus is isolated and the amino acid residue at position 226 has been implicated in this, though other residues may be involved (Rogers and D'Souza, 1989; Aytay and Schulze, 1991; Nobusawa *et al.*, 1991). Binding to host cell occurs rapidly after attachment and involves some irreversible changes in the HA that do not occur when HA binds to red blood cells to produce haemagglutination (Clague *et al.*, 1991).

1.3.2 Fusion

Proteolytic cleavage of the HA molecule into HA1 and HA2 is required for virus particle entry into the host cell. This cleavage occurs mainly intracellularly, mediated by trypsin-like enzymes at low pH and thus links fusion capacity with capacity of the virus to infect (Garten *et al.*, 1981). At pH <6 which is the pH of endocytic vesicles in the host

cell, HA mediates fusion with the host cell membrane (Huang *et al.*, 1981a,b). M protein is also solubilised at acidic pH which may assist uncoating (Zhirnov, 1990) but HA alone is capable of fusion with host cells. The kinetics of fusion mediated by influenza virus HA appears to be influenced by both the molecular structure of HA protein (Pritzen *et al.*, 1990) and by non-specific physical properties of the membranes involved (Clague *et al.*, 1991). Thus after cleavage the 3 dimensional structure of HA molecule undergoes significant conformational change where the amino terminus of HA2 is found 21 Å from the carboxyl terminus of HA1 having moved outward away from the interior stalk, making this portion of HA2 more available for interaction with the surface of the infected cell. The sequence of about 20 amino acids at the newly created amino terminus of HA2 is the most highly conserved sequence of the HA molecule and exhibits the sequence generated when other virus fusion proteins are activated by proteolytic cleavage as in Paramyxovirus F1 fusion protein (Richardson *et al.*, 1980). Similarly, a number of other virus fusion glycoproteins have been found to have repeat sequences adjacent to the hydrophobic regions at the amino terminus as in influenza HA2 (Chambers *et al.*, 1990). It is now known that the nature of interaction between HA and host cell membrane is by pore formation in an effort to increase pore conductance of the cell membrane which at the initial stage may involve only a few HA molecules (Spruce *et al.*, 1991).

1.3.3 Virus RNAs in the Infected Cell

In the infected cell, the eight segments direct the synthesis of two forms of RNAs of positive polarity, capped polyadenylated mRNA molecules and uncapped non-polyadenylated cRNA molecules which serve as template for vRNA synthesis (Fig. 3). Characterisation of tne terminal sequences of these three RNA species has been the subject of intense investigation in order to define the relationships between the virus genome, the polypeptide products it encodes and the host cell (McCauley and Mayh, 1983).

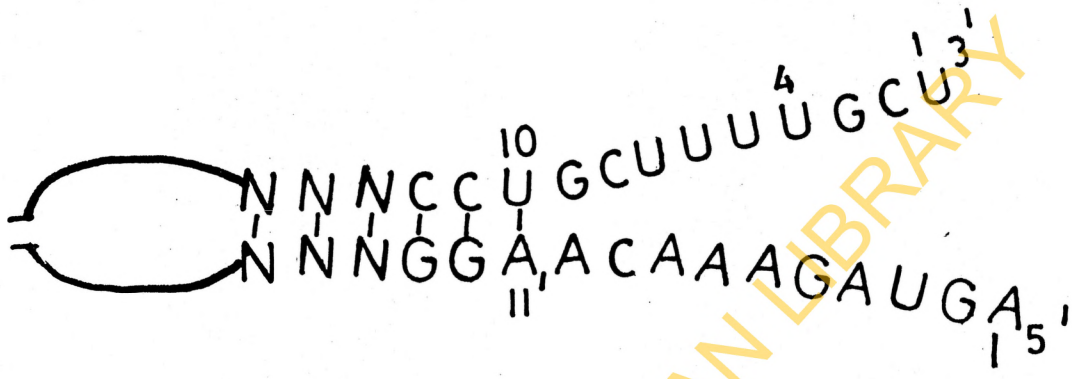
The nucleus is the site of all viral transcription (Hertz *et al.*, 1981; Jackson *et al.*, 1982). Most of the NP enters the nucleus as part of the RNP complexes by an active process within 10 minutes of the virus penetrating the cell (Martin and Helenius, 1991). A nuclear location signal has been described for PB1 protein (Nath and Nayak, 1990), PB2 protein (Mukaigawa and Nayak, 1991) and NP (Davey *et al.*, 1985).

The transcriptional mechanism of influenza viruses is unique requiring special cooperation of virus and cell activities. Their transcription is thus mediated mainly by a virus-coded RNA-dependent RNA polymerase with similar functions to that of RNA transcriptases of other negative strand RNA viruses. Synthesis of mRNA requires the transcription of cell DNA by DNA dependent RNA polymerase II (Lamb and Choppin, 1977) and only RNP complexes present in the nuclear matrix fraction of cells can synthesise vRNA (Lopez-Turisco *et al.*, 1990). Influenza virus transcriptase is unable to initiate mRNA synthesis or modify the 5' termini of mRNA molecules by capping and methylation. Hence, it has to be primed by capped and methylated 5' termini supplied by the action of cellular mRNA producing the enzyme RNA polymerase II (Mahy *et al.*,

1972).

Both the 3′ and 5′ terminal sequences are involved in the initiation of transcription (Fodor *et al.*, 1994). The 12 nucleotides at the 3′ end and the 13 at the 5′ end are conserved in influenza A viruses, they are partially complementary and form a panhandle structure in isolated virion and infected cells (Honda *et al.*, 1987; Hsu *et al.*, 1987; Fig.4).

Fig. 4: An RNA forked model for initiating influenza virus transcription. Adapted from Fodor *et al.* (1994).



This panhandle structure is a cis-acting signal for polyadenylation (Luo *et al.*, 1991) and it is required for endonuclease activity of RNA polymerase complex (Hagen *et al.*, 1994). The 9-12 nucleotides of influenza A virus vRNA promoter at the 3′ end of vRNA segment and the 13 nucleotides at the 5′ end are involved in RNA polymerase binding (Fodor *et al.*, 1993; 1994) because of one or more binding sites for polymerase subunit(s) on the 5′ single stranded region and on the duplex region of the panhandle which is an RNA-forked structure partly double stranded at residues 10-13 or 15 and partly single stranded at the 2 ends. In addition to base pairing, the duplex region is also stabilised by protein-RNA or protein-protein interactions which are dependent on the RNA polymerase subunits. The involvement of the panhandle structure suggests novel mechanisms for the control of influenza A virus mRNA and cRNA synthesis which differs in both initiation and termination steps. Whereas mRNA is capped, prematuredly terminated and polyadenylated, cRNA is a full-length copy of vRNA and is neither capped nor polyadenylated. During viral life-cycle, transcription initiation switches from mRNA synthesis to predominantly cRNA synthesis and the presence of an RNA polymerase binding site in the 5′ end sequence of the RNA forked structure is thought to facilitate the switch. The three P proteins form a complex in the cell regardless of being part of RNPs or not (Akkina *et al.*, 1987, Digard *et al.*, 1989). Within this complex it is the basic polypeptide PB2 that recognises the 5′ terminal cap structures of newly synthesised cellular RNA produced by cellular RNA polymerase II (Ulmanen *et al.*, 1983). For initiation of mRNA synthesis, PB2 subunit of RNA polymerase generates capped RNA

primers by endonucleotide cleavage of host cell pre-mRNA in the nucleus of infected cell at purine residues 10-14 nucleotides from the 5′ methylated cap. This produces capped fragments with 3′ terminal hydroxyl groups which can serve as primers without dephosphorylation for initiating viral mRNA transcription. This is followed by incorporation of a guanosine residue into the 3′ end of capped RNA primer mediated by virus transcriptase with the penultimate base in each vRNA template segment being a complementary residue (Fig. 5; Plotch *et al.*, 1981; Ulmanen *et al.*, 1983). Cell fragments used as primers seem to be heterogenous in nature but cellular RNAs having the sequence GCA immediately before the cleavage site are preferred (Lamb *et al.*, 1981). A specific interaction of the RNA polymerase with the 3′ end and the 5′ end of vRNA is required for initiation activity of PB2 (Hagen, *et al.*, 1994) and for initiation of mRNA synthesis. Steric hinderance inevitably causes premature termination of mRNA synthesis at the U stretch near the 5′ end as the RNA polymerase remains attached to the binding site in the 5′ end sequence of template vRNA during transcription. Furthermore continued synthesis by RNA polymerase leads to the synthesis of poly (A) tail as a result of reiterative copying of the same U stretch. On the other hand, a regulatory protein, e.g. NP (Beaton and Krug, 1986) or NS1 (Hatada and Fukuda, 1992) may modify the panhandle structure or binding of RNA polymerase which would prevent PB2 from further generating capped RNA primers. This thus allows primer-independent initiation of cRNA synthesis by RNA

Fig. 5. Priming of influenza virus mRNA transcription by host cell mRNA.

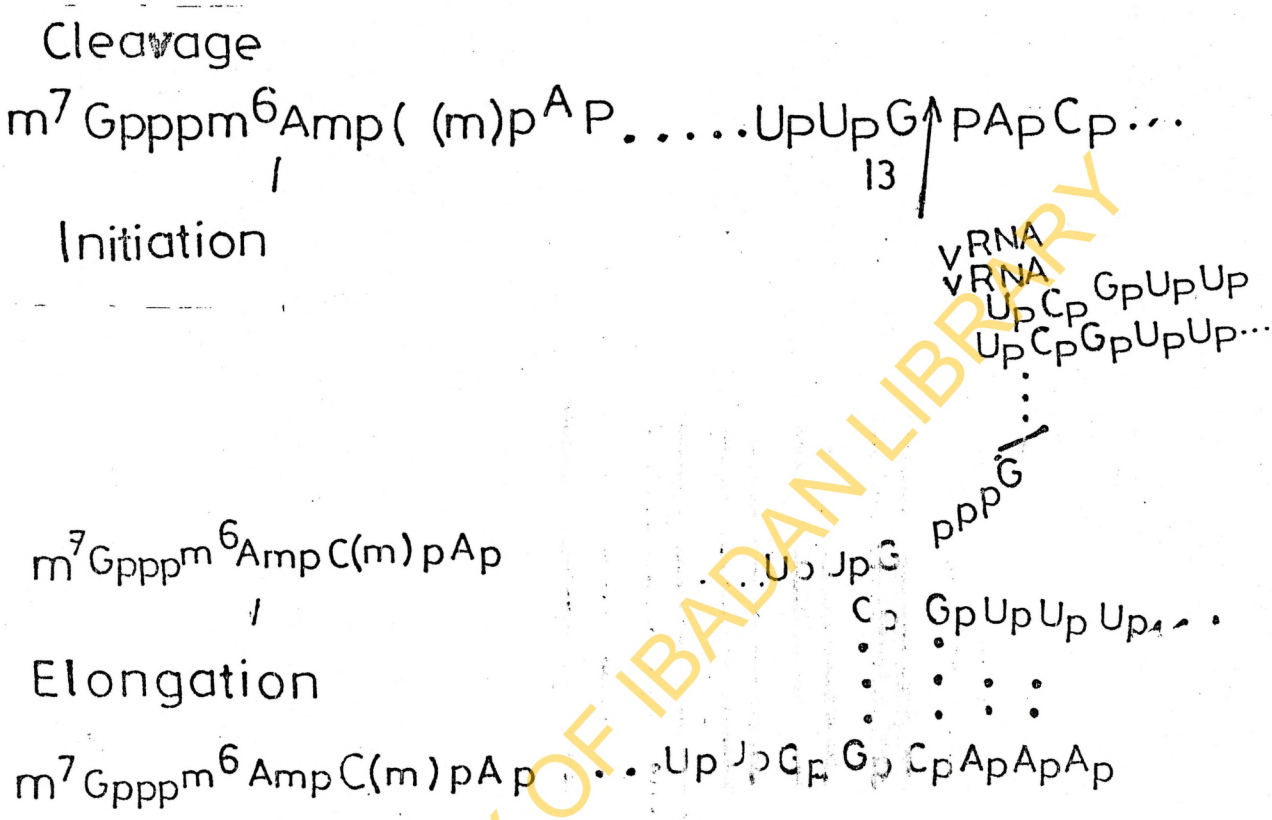


Fig. 5. Priming of influenza mRNA transcription by host cell mRNA. Adapted from Ulmanen et al (1981, 1983) Plotch et al (1981).

polymerase acting either on the modified panhandle or on a single 3' terminus. Moreover, the absence of RNA polymerase from the 5' end sequence would result in the synthesis of full-length cRNA.

PB2 also plays some role in chain elongation (Yamanaka *et al.*, 1990). The RNA polymerase complexes on their own synthesise only short RNAs but when NP is involved in the RNP complex, chain elongation is observed, suggestive of a major role of NP in chain elongation and stabilisation of the RNA template (Honda *et al.*, 1988). The other basic protein PB1 catalyses the initiation of transcription and continuation of mRNA synthesis as the P protein complex moves along the nascent RNA with PB1 at the leading edge and PB2 at the trailing edge (Braam *et al.*, 1984). Chain elongation is also modified by PB1 and transcripts terminate at sites 15-22 bases from the end of their templates where oligo U sequences are used repetitively as templates for the synthesis of 3' terminal poly (A) tract.

1.3.4 Transcription and Replication

Virus specific transcription occurs in three stages. The first stage is the primary transcription of infecting virus genome which results in the generation of small equimolar amounts of all mRNAs as described above. These virus encoded mRNAs are partial transcripts and secondary transcription is required for production of full length template non-polyadenylated (A-) RNAs needed for the synthesis of RNA to be incorporated into newly replicated virus (Krug, 1983; Lamb and Choppin, 1983). The second stage is early secondary transcription which occurs between half an hour to 2 hours after infection and

depends on virus protein synthesis for cRNA production. Full length cRNA is transcribed only from the infecting virus genome from all the 8 segments in equimolar amounts by virus polymerase enzyme complex and transcription is maximal before 4 hours post infection (Hay *et al.*, 1977; Barret *et al.*, 1978; Inglis *et al.*, 1978; Smith and Hay, 1982). Regulated synthesis of vRNA from the template cRNA also occurs in early secondary transcription stage (Fig. 6). Synthesis of template RNAs to be used in vRNA synthesis is dependent on continued production of viral proteins and it is initiated independently. Like vRNA, cRNA appears to be synthesised in the nucleus, occurring at fixed sites in the nuclear cage (Jackson *et al.,* 1982; Beaton and Krug, 1984). Transcripts of vRNA segments 5 and 8 are amplified and maximum mRNA synthesis of these segments occurs at the same time. While the synthesis of cRNA is not regulated, regulation of vRNA synthesis appears to be at the transcription level with the newly synthesised vRNA being transcribed only for a short period (Barret *et al.,* 1978; Smith and Hay, 1982).

The early secondary stage progresses to late secondary transcription, requiring protein synthesis and some host cell factor to enable transcription to continue past the termination site used in mRNA synthesis. Synthesis of vRNAs and mRNAs of segments 4, 6 and 7 are amplified while those of segment 8 is reduced but those of segment 5 remain high. Transcription of segments 1-3 is at a low level throughout infection but synthesis of vRNAs of these segments is amplified a little in late secondary transcription (Fig. 6).

Fig. 6. Scheme for influenza virus genome replication.

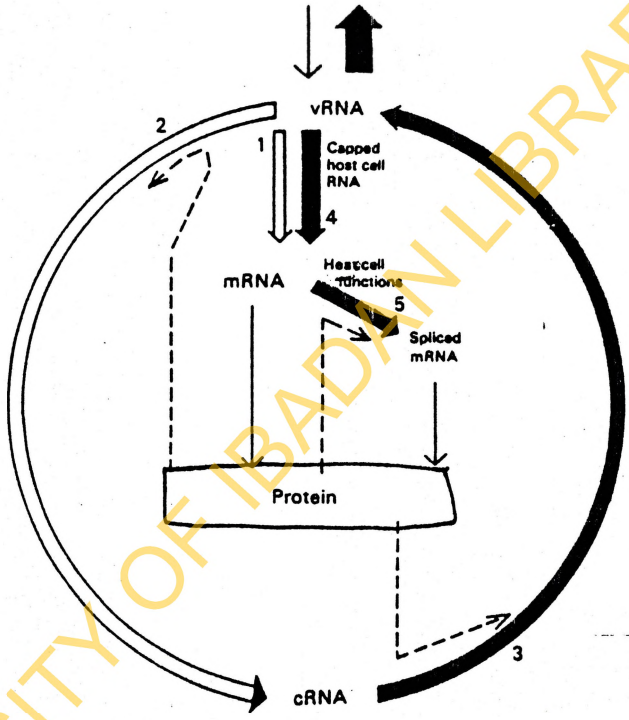


Fig. 6. *Scheme for influenza virus genome replication*

⟹, Transcription from input genome RNA; ➡, transcription from cRNA or newly synthesized vRNA; ------> necessary interactions. Step 1: primary transcription, which occurs in the absence of protein synthesis but requires host cell transcription. Step 2: cRNA synthesis from input genome RNA, and step 3: selective and amplified vRNA synthesis directed by cRNA, both require on-going protein synthesis. Step 4: transcription of newly synthesized vRNA to form mRNA requires host cell caps. Step 5: modification of mRNAs 7 and 8 by splicing requires protein synthesis and undefined host cell functions.

Adapted from McCauley and Mayh (1983).

Maximum production of M2 occurs late in infection and requires protein synthesis (Valcarcel *et al.*, 1991). However, differences in the virus-host system and methods of study may reflect the differences observed in the regulatory control of synthesis. The mRNAs of influenza virus are not all transcribed from collinear nucleotides. Segment 7 contains three reading frames (Ulmanen *et al.*, 1991). The one for M1, the longest structural protein occupies 75% of the segment length and the transcribed mRNA is colinear. The second reading frame encoding the smaller M2 non-structural protein overlaps the M1 reading frame by 14 amino acids and the mRNA is produced by splicing in that it contains interrupted regions. The third reading frame encoding M3 is also spliced and codes for 9 amino acids identical to those of M1 at the carboxyl terminus but no protein has been found in the infected cell. Similarly segment 8 contains a second reading frame encoding NS2 protein which overlaps the NS1 reading frame by 70 amino acids. NS2 mRNAs are produced by splicing, removing the bulk of NS1 sequences and the spliced RNAs are read in the NS1 reading frame (Lamb and Choppin,.1983).

1.3.5 Viral Protein Synthesis (Translation)

New viral proteins are detectable in infected cells within an hour of infection, associated with a decrease in host protein synthesis thereafter (Skehel, 1972). Temporary control of viral protein synthesis has been established and shut off of host protein synthesis is neither rapid nor complete. NS and NP are preferentially synthesised early while HA, NA and M proteins show a marked increase late in infection with the pattern of protein synthesis closely mimicking that of mRNA and vRNA (Skehel, 1972; Hay *et al.*, 1977).

NS1 may be involved in post-transcriptional processing of late proteins (Hatada *et al.*, 1990). Like other glycoproteins, HA and NA follow the maturation pathway of cell glycoproteins and are translated on membrane-fixed ribosomes. They possess hydrophobic domains and signal sequences and they are co-translationally inserted into the host cell membranes. Both proteins also undergo glycosylation during transport to the cell surface (reviewed by McCauley, 1987). HA oligosaccharides are further modified, including trimming and formation of complex oligosaccharides in the Golgi complex (reviewed by Nayak and Jabbar, 1989). M and most other viral proteins are translated on free ribosomes. After synthesis, M protein becomes quickly associated with both plasma and nuclear membranes (Meir-Ewert and Compans, 1974; Oxford and Schild, 1975; Hay and Skehel, 1975). It is thought that M protein plays a role in terminating mRNA synthesis (Hankin *et al.*, 1989) and promotes the export of vRNA from the nucleus (Martin and Helenius, 1991). M protein may be phosphorylated, thus explaining the heterogeneity observed in this protein (Gregoriades, 1984; Gregoriades *et al.*, 1990). On the other hand, M2 protein exists in infected cells as a homotetramer composed of two disulphide-linked diamers which are held by non-covalent interactions. It has a hydrophobic domain and becomes inserted into the plasma membrane (Zebedee *et al.*, 1985; Holsinger and Lamb, 1991). Studies on the antiviral activities of amantadin at low doses through its action on M2 have revealed that M2 acts as a tetrameric proton translocation channel that regulates pH in the endoplasmic reticulum and Golgi network. It is involved in maintaining the correct conformation of HA in transit to the cell surface (Sugrue *et al.*, 1990b; Sugrue and

Hay, 1991). M2 has also been found in small quantities in virions suggestive of its action in the control of the interior pH of the viral particle (Zebedee and Lamb, 1988).

Synthesis of PB1, PB2 and PA continues at a low rate throughout infection. All the three P proteins are found in very close association with the nucleus of infected cell while PA is also found in the cytoplasm, their nuclear location being consistent with their role in primary transcription which is a nuclear event. However, the continued presence of PA in the cytoplasm is suggestive of another role such as in vRNA synthesis. NP migrates promptly to the nucleus prior to the appearance of HA and NA in the cytoplasm. It is subsequently found in the cytoplasm and finally at the periphery of the cell (Maeno and Kilbourne, 1970). Along with the P proteins, NP is found in nucleocapsids (RNPs) which contain vRNA and remains in the nucleus but its nuclear location is not dependent on complexing with RNA or P proteins. Phosphorylation of a proportion of NP occurs soon after synthesis, associated with transport to the nucleus while non-phosphorylated NP remains in the cytoplasm. The ratios of phosphorylated and non-phosphorylated NP seems to change throughout infection suggesting a possible nuclear location signal (Almond and Felsenreich, 1982). The correct phosphorylation of NP appears to play a role in replication by integrating the components of the virus particle. A cell protein kinase has been implicated in this process (Kistner *et al.*, 1989). NS1 is synthesised early and in large quantities. Both NS1 and NS2 have been located in the nucleus and the cytoplasm (Breidis *et al.*, 1981; Greenspan *et al.*, 1985). However, monoclonal antibodies have detected NS2 at a low level in the virus particle while NS1 has not been detected and it has thus been suggested that NS2 may be a structural protein (Richardson and Akkina, 1991)

1.3.6 Maturation and Assembly

As proteins are synthesised and accumulated assembly of virus particle occurs. The initial event is the insertion of HA and NA into the cell's plasma membrane preceded or accompanied by displacement of host proteins. M protein then accumulates beneath the HA and NA. This process is thought to have a major role in determining the rate of virus assembly (Downie, 1984). M, NP and presumably other viral structural proteins reach the membrane directly by diffusion. Results of chemical cross-linking studies suggest that M protein may interact on a 1:1 basis with NP in the virion (Dimmock *et al.*, 1989). This may be relevant to the formation of virus particles prior to budding through the plasma membrane. HA is not directly involved in interaction with RNPs during assembly as its molecules behave as separate molecules in labelling experiments under the electron microscope (Patterson *et al.*, 1988). However, recent studies have indicated a possible interaction between HA, M and NP genes in the control of filamentous as opposed to spherical virus particles (Smirnov *et al.*, 1991) particularly fresh isolates.

1.3.7 Budding and Release

The two modules that comprise influenza virion are assembled in different locations; the nucleocapsid is assembled within the nucleus and the envelope is assembled at the cell surface membrane (Kingsbury, 1990). Budding of virus particles occurs very rapidly following assembly of M and RNPs as seen in electron microscopic studies (Patterson *et al.*, 1988). Unlike most other enveloped viruses, influenza viruses do not acquire neuraminic acid during assembly and budding possibly because an active NA is

already incorporated in the virus particles. NA is important in the final release and detachment of the virus . It facilitates virus release not only at the cell surface but also by destroying cellular neuraminic acid (reviewed by Griffin *et al.*, 1983).

1.4 Abortive/Inefficient virus replication and Defective Interfering Viruses

The productivity of influenza virus infection as regards yield of infectious virus represents a gradient ranging from the translation of a limited number of viral proteins (Minor and Dimmock, 1976) to replication with release of a relatively high ratio of infectious to defective virus particles (Choppin, 1969). The efficiency of infective virus production is dependent on virus-host cell interaction, multiplicity of infection, presence of defective interfering virus and environmental factors (Kilbourne, 1987). A number of factors may cause abortive or inefficient replication. These include impairment of RNP transportation from the nucleus to the cytoplasm, defective post-translational cleavage of HA, defects in M protein synthesis late in infection and defective budding of virus from plasma membrane (Ghandi *et al.*, 1971; Inglis and Brown, 1984; Takeura *et al.*, 1984).

Defective interfering particles (DIP or DI particles) are generated during the normal replication of influenza viruses described above. DI particles are also associated with most RNA and DNA animal and plant viruses as well as bacteriophages. Generally, DI particles lack part of the genome, requires the presence of a helper virus to replicate, interfere with the growth of homologous helper standard viruses and are packaged with usual virus proteins (Huang and Baltimore, 1970; Bean *et al* , 1985). The extent of their interference is thought to vary according to the cell type and the virus involved (Cole and

Baltimore, 1973). A reduction in the ratio of plaque forming unit to haemagglutinin unit (PFU:HAU) is associated with the presence of DI particles. In influenza virus infections, three types of incomplete particles have been observed. These are, (i) heat inactivated particles (von-Magnus, 1954), (ii) subgenomic particles lacking one or more genome segments but which do not interfere with the replication of the standard virus and are termed non-interfering particles-DNI (Hirst and Pons, 1973; Carter and Mayh, 1982a, b) and (iii) genetically defective particles capable of interference (classical DI particles). These lack the three largest RNA segments and contain DI RNAs which arise from standard influenza virus genes by deletion of the P genes (Crumpton *et al.*, 1978; Nayak *et al.*, 1978; Pons, 1980). The DI RNAs contain the 5' and 3' ends of the parent segments. Most of them are from segment 1 but they could arise from any segment by simple internal deletion, double deletion, double deletion and insertion or by containing sections from two different segments (Nayak *et al.*, 1982; Fields and Winter,1982; Jennings *et al.*,1983). They are not reproducibly distinguishable from standard viruses by their morphology or physical characteristics but are variable in their content of the viral structural proteins HA, NA, M and NP (reviewed by Nayak *et al.*, 1985). DI particles have reduced amounts of viral RNA usually involving the polymerase (P) genes and contain small RNA segments which are specific and negative stranded. The RNAs are truncated P genes. As with other negative strand viruses, the polymerase complex may switch strands during RNA synthesis and switching from the template to the nascent RNA strand results in RNAs with complementary ends. Such DI particles are referred to as

copyback type having been generated by "polymerase jumping" from the 5′ end to the 3′ end of RNA and the looped tertiary structure of RNPs brings these ends close together (Nayak *et al.*,1982; Jennings *et al.*, 1983; Holland, 1990).

1.5 Encapsidation and Packaging of Virus Genome

Influenza mRNAs are not encapsidated but the negative strand genome segments and the positive strand antigenome segments required for RNA replication are complexed with the nucleocapsid structure units. It is presumed that the sites for initiating encapsidation are in the terminal sequences that are not copied during mRNA transcription (Kingsbury *et al.*, 1987). As in other negative strand viruses, influenza virus packages negative sense RNAs but it needs to package more than one segment. Two possible mechanisms have been proposed for packaging all the eight segments into the virus particles. The first is random packaging (Hirst, 1962; Lamb and Choppin, 1983; Luytjes *et al.*, 1989; Muster *et al.*, 1991). The second possible mechanism is specific association of virus segments where it has been observed that all segments are present in the released virus in equimolar amounts (McGeoch *et al.*, 1976; Lubeck *et al.*, 1979; Smith and Hay, 1982; Carter and Mayh, 1982; Ye *et al.*, 1989; Skorko *et al.*, 1991). Duhust and McCauley (1991) have also reported a specific packaging mechanism for influenza A viruses.

1.6 EPIDEMIOLOGY

1.6.1 Historical Perspective

The history of human influenza dates back to long before 412 B.C. when Hippocrates recorded one of the influenza epidemics occurring at that time. Since then epidemics of varying degree of severity had occurred relatively frequently and at regular intervals with occasional disappearances (Noble, 1982). The virus responsible for human epidemics was first isolated in 1933 by laboratory infection of ferrets with human nasal washings following 15 years of research to find the causative agent of the influenza pandemic of 1918 ("Spanish Flu") which within four months resulted in 25 million deaths (Smith *et al.*, 1933; Webster and Kawaoka, 1994). Since then influenza had remained the most serious unconquered acute threat to man (Webster *et al.*, 1992) giving rise to pandemics in 1957 ("Asian flu") and 1968 ("Hong Kong flu") followed by the "Russian flu" in 1969 originating from China between which epidemics of serious proportions had occurred almost annually. Human pandemics or major epidemics occur when there is antigenic shift of the HA particularly if this is associated with a change in the NA (Nicholson, 1994).

Influenza epidemics also occurred in animals. In 1956, Heller, Espmark and Viriden found antibodies to soluble antigens of influenza A virus in the sera of horses convalescent from an influenza-like illness following which an influenza A virus, equine-1, A/Equine/Prague/1/56 (H7N7) was isolated in Czechoslovakia from infected horses (Soirnova *et al.*, 1985). In 1963, equine influenza equine-1 occurred in England while in America an extensive outbreak occurred leading to the isolation of a strain of the virus

very distinct from the Czechoslovakia and English strains (Hoyle, 1968) and it was termed equine-2, A/Equine/Miami/63 (H3N8). It is notable that concurrent respiratory disease in horses was frequently remarked in the 18th and 19th century epidemics in man as the epidemic of 1762 was preceded by "horse colds" in 1760 (Kilbourne, 1987). No fewer than nine recorded epidemics of influenza throughout the world between 1693 and 1873 were associated with epidemics of what appeared to have been equine influenza (Hirsch, 1883) although now-a-days independent outbreaks of influenza in horses and man occur. It is thus probable that man and horses must have traded or reassorted viruses in the 17th, 18th and 19th centuries being in more closer proximity then that coccirculation of viruses of different subtypes might have been responsible for epidemics characterised by variable virulence and occasional pandemics when new subtypes were introduced. Thus the variable severity of such pandemics may have reflected partial immunity of the populations to either HA or NA antigens and variation in intrinsic viral virulence due to mutations or seasonal differences in the occurrence of influenza (Kilbourne, 1987).

Similarly swine and human viruses were known to be serologically related and that human strains could infect swine (Hoyle,1968) and vice versa. Three subtypes of influenza A virus infect pigs, the classical H1N1 which are descendants of A/Swine/Iowa/15/30, the avian-like H1N1 and human-like H3N2 (Nicholson, 1994). Influenza in swine was first observed in the United States of America during the 1918 human influenza pandemic. The isolation of swine influenza virus (A/Swine/Iowa/15/30, H1N1) by Shope in 1931 and retrospective serological studies indicated that the swine virus was antigenically similar to the type A influenza virus responsible for the 1918

human pandemic. The pathogenicity of the swine virus for man was confirmed by the isolation of the swine H1N1 virus from military recruits at Fort Dix in 1976 following influenza infection in these recruits and subsequent isolations of antigenically and genetically indistinguishable isolates from a man and a pig on the same farm in Winsconsin (Hinshaw *et al.*, 1978).

In addition to man and animals, influenza virus also infects birds and had caused an outbreak in harbour seals (Webster *et al.*, 1981). The causal agent of this outbreak was shown to be closely related to the fowl plague virus A/FPV/Dutch/27 (H7N7), a highly virulent influenza virus of chickens not previously found in mammals (Webster *et al.*, 1981). Fowl plague was first described in Italy by Perroncito in 1878 and shown to be caused by a virus by Centanni and Savunozzi in 1900 (Stubbs, 1965). The fowl plague virus was shown to be genetically and antigenically similar to human influenza A virus being only different in its host range which is limited to avians (Schaffer and Munk, 1952). Several isolates of influenza A viruses have since been reported in ducks and other avians. Influenza virus also infect primates, mink and whales. In seals, 2 strains have been isolated, A/Seal/Mass/1/80 (H7N7) and A/Seal/Mass/2/80 (H4N5), whose 8 segments are closely related to those from avian influenza viruses (Hinshaw *et al.*, 1984) suggesting the importance of these isolates in the ecology of influenza viruses. Influenza viruses, presumably of avian origin, have also been isolated from minks (H10N4) and from pilot whales (H3N2 and H13N9) derived from sea gulls (Hinshaw *et al*, 1986).

1.6.2 Antigenic variation

Influenza A viruses undergo two types of antigenic variation termed antigenic drift and antigenic/genetic shift which involve minor and major changes respectively in their HA and NA genes. These changes occur by genetic reassortment. When this takes place between human strains and other species strains of the virus particularly those of birds, antigenic shift occurs (Webster *et al.,* 1982). This reassortment is possible because of the peculiar structure of influenza virus genome which is segmented into 8 distinct single-stranded pieces. Antigenic shift occurs when novel influenza A viruses emerge following replacement of an HA or NA subtype with a new variant different from those of the previously prevalent viruses (Pereira *et al.*, 1967; Webster and Laver, 1975). The introduction of new subtypes of viruses into human populations through antigenic shift results in pandemics. Thus, since the first isolation of human influenza A subtype H1N1 in 1933 (Smith *et al.*, 1933) antigenic shifts have occurred in 1957 when the H2N2 subtype viruses of Asian influenza replaced the H1N1 subtype, in 1968 when the Hong Kong (H3N2) virus emerged and in 1977 when the H1N1 virus reappeared (Zhdanov *et al.*, 1978) and in equines when the H3N8 virus emerged in 1963 (Hoyle, 1968). On the other hand minor changes can also occur within a subtype, which when they accumulate over a period of time could cause the emergence of different strains resulting in antigenic drift (Webster *et al.*, 1982; Kawaoka *et al.*, 1989; Migunova *et al.*, 1990; Endo *et al.* 1992). Persistent and recurrent epidemics which occur between pandemics are often caused by antigenic drift.

1.6.3 Origin, Evolution, Genetic relatedness and Ecology of influenza viruses

The evolution and ecology of influenza A viruses have recently been reviewed by Webster *et al.*, (1992) and a number of studies have been carried out to indicate the origin and genetic relatedness of influenza virus genes (Bean, 1984; Kawaoka *et al.*, 1989a; Mandler *et al.*, 1990; Gorman *et al.*, 1990a,b, 1991; Bean *et al.*, 1992; Guo *et al.* 1992). Available body of evidence from these studies strongly suggests that all the genes of influenza viruses currently circulating among mammals and birds originated from avian influenza viruses although separate host-specific gene pools exist. The general structural features, genome organisation and sequence analyses of influenza A, B and C viruses indicate that they originated from a common ancestor distinct from other negative strand viruses (Desselberger *et al.*, 1980, Smith and Palase, 1989) although influenza A and B viruses are more similar to each other in genome organisation and protein homology than influenza C viruses which suggests that the C viruses diverged from their common ancestor long before the A and B viruses split (Smith and Palase, 1989). Influenza B and C viruses are thought to originate, like the A viruses, from avian viruses but unlike A viruses, influenza B and C viruses do not reassort to leave viable progenies, having reached a state of evolutionary equilibrium with their hosts.

The origin of influenza viruses addresses two related but distinct issues of the original host of the first influenza virus and the host(s) in which the nearest common ancestors of each of the virus gene segments could be found. Phylogenetic analyses have provided strong evidence that the waterfowls may be the original hosts of the first influenza virus (Webster *et al.* 1981, 1992) as influenza A viruses seem to be long

established pathogens of wild aquatic birds and are more transient in other hosts considering their high degree of adaptation to avian species, the vast genetic diversity of avian virus subtypes and the evolutionary stasis of avian virus proteins. However, considerable reassortment of non-avian with avian viruses has occurred repeatedly over the evolutionary history of influenza viruses. Thus, it is possible that genes or whole genome can be transmitted to new mammalian hosts, displace previous strains and evolve independently from their avian ancestors as demonstrated in pigs in Europe (Scholtissek *et al.* 1983) and more recently in horses in China (Guo *et al.* 1992a,b; Shortridge *et al.*, 1995).

Studies on the genetic relatedness of influenza virus genes have indicated the evolutionary relationships among influenza A viruses isolated from different hosts in diverse geographical locations (Kawaoka *et al.* 1989a; Okazaki *et al.*, 1989; Nakajima *et al.*, 1990a,b; Gameline *et al.*, 1989, 1990; Altmuler *et al.*, 1991; Ito *et al.*, 1991; Bean *et al.*,1992). The NP genes have evolved into five major host specific lineages. These are (i) an ancient Eq/Prague/56 (equine-1 viruses), (ii) recent equine viruses (equine-2) related to Eq/Miami/63, (iii) human viruses and classical swine viruses related to Swine/Iowa/15/30, (iv) H13 gull viruses and (v) all other avian viruses (Scholtissek *et al.*, 1985; Webster and Kawaoka, 1994). None of the available phylogenies (Fig. 7) for all the eight gene segments shows identical topologies suggestive of the effects of past reassortment events and possible extinction of some viruses on evolutionary patterns of individual genes (Webster *et al.*, 1992). The Eq/Prague /56 virus shows a very distant

relationship to other virus lineages indicating that it is the most divergent and possibly the oldest of the non-avian influenza A virus lineages. The NP and PA gene phylogenies are very similar indicating that they share a common evolutionary history in each host and have probably not reassorted independently. The M gene phylogenies differ significantly from those of NP and PA in that equine-2 M genes are probably recently derived from the North American avian viruses contrary to the much older equine-2 NP and PA genes. Moreover, the H13 gull M gene seems to be closer to Eq/Prague/56 M gene than the gull NP and PA genes while the gull PB2 gene on the other hand shows a more recent origin than even the gull NP, PA and M genes.

The origin of the H13 gull NS gene like the M gene is also older than the gull NP, PA, PB1 and PB2 genes (Treanor *et al.*, 1989). The earliest avian virus isolate, the pathogenic fowl plague virus NS gene appears to be derived from an older lineage than the break between the Eurasia and American avian, human and classical swine viruses while the recent equine-2 virus NS genes are more recent in origin and seem to be derived from the American avian virus group. Unlike the other internal genes however, the PB1 gene lineages are less congruent. Human PB1 genes originated from three different sources, the origin of the gull PB1 gene is very recent while the recent equine-2 virus PB1 gene has a much older origin like the NP and PA genes.

The human HA genes currently circulating appear to have diverged rapidly from their 1965 progenitor which caused the 1968 pandemic having originated from an avian virus (Bean *et al.*, 1992). The equine-2 H3 HA genes appear to have diverged much earlier from an avian ancestor while the swine H3 HA genes are derived from both human

Fig. 7: Phylogenies of influenza virus genes: The horizontal distance is proportional to the minimum of nucleotide differences needed to join the gene sequences. Vertical lines space the branches while the arrow at the left of each tree represents the node the connects influence is virus homolog. NAm refers to North-American avian viruses and OW refers to old World or Eurosian avian viruses. Adapted from Webster et a, [1992].

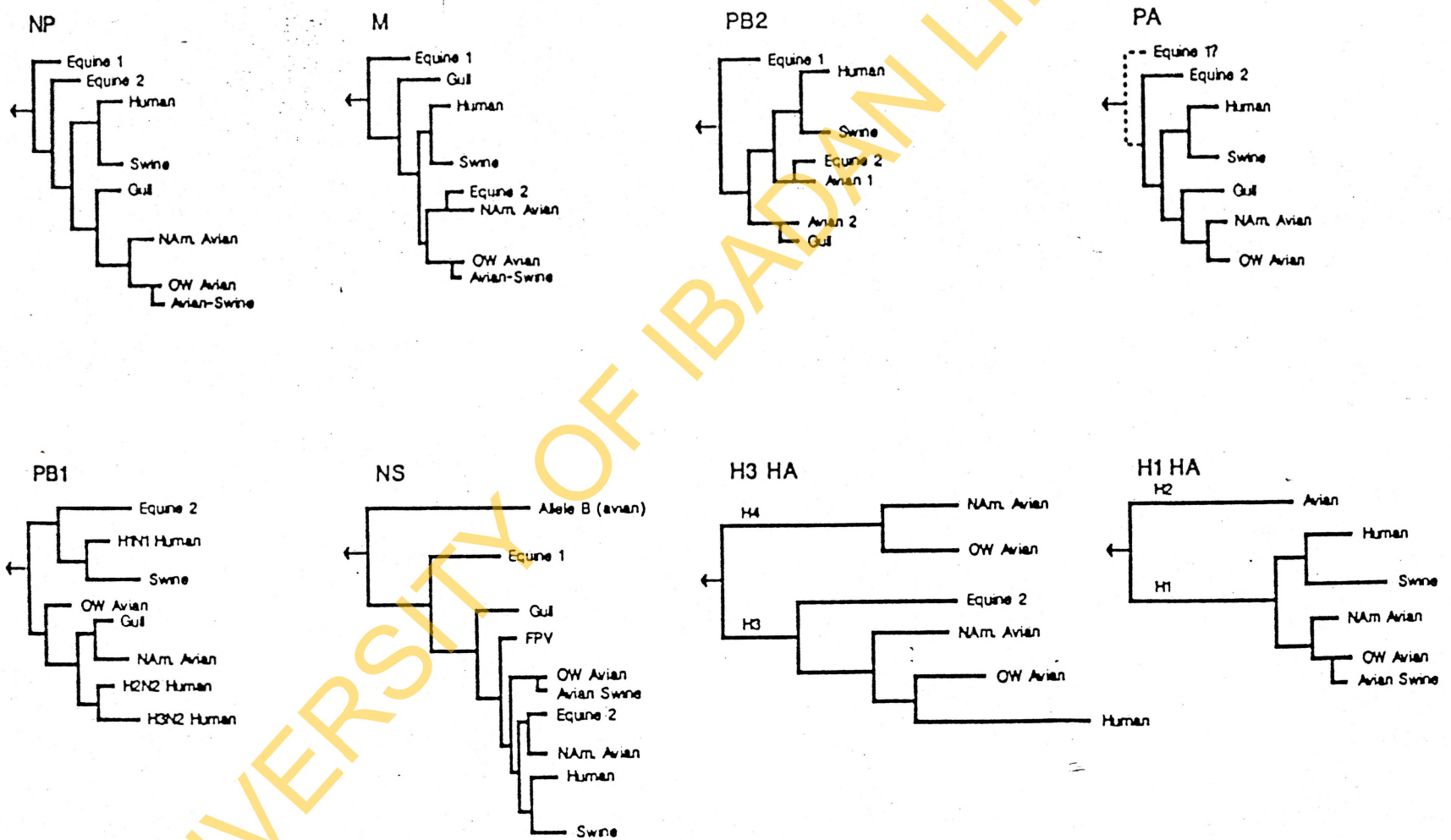


Fig. 7. Phylogenies of the 8 gene segments of influenza A virus.

and avian viruses. (Castrucci *et al.*, 1993). Thus pigs may serve as a "mixing vessel" in the transmission of avian influenza viruses or their genes to the human gene pool. Phylogenetic analyses also show that the NP, PB2, M and NS genes of Eq/Prague/56 virus are consistently closer to influenza B virus homologs further reinforcing the much earlier divergence of this virus from avian viruses than other non-avian viruses.

Influenza is a viral disease in which continued evolution of the virus is very important in producing annual epidemics and occasional pandemics in humans and possibly other mammals (Webster *et al.*, 1992). The evolutionary patterns of influenza A, B and C viruses show that the three virus types evolved through fundamentally different mechanisms. The evolution of the C virus is different from that of A virus. It is characterised by co-circulation of many strains of the virus giving rise to multiple evolutionary lineages with much lower rate of nucleotide substitutions than in the mammalian influenza A viruses. This pattern also occurs in avian influenza A viruses while the evolution of the B viruses is intermediate between those of A and C viruses (Buonagurio *et al.*, 1986; Yamashita *et al.*, 1988). Both antigenic drift and genetic shift occur in human, swine and equine influenza A viruses and contribute significantly to virus evolution which is dependent on periodic introductions of gene segments or entire genomes from avian influenza virus gene pool. This evolutionary process is most prominent in the surface glycoproteins of the A viruses but also occurs in each of the eight gene segments of both A and B viruses resulting in genetic variations in the viruses due to accumulation of molecular changes in the eight gene segments which can occur through different mechanisms. These mechanisms include point mutation (antigenic drift), gene reassortment

(genetic shift), defective interfering particles (DIP) and RNA recombination (Webster *et al.*, 1992).

Ecology deals with the interrelationships of organisms and their environment and influenza viruses from different hosts in diverse geographical locations provide a unique opportunity for examining the ecology and evolution of an RNA virus. Non-pathogenic avian influenza viruses are ubiquitous particularly in aquatic birds (Alexander, 1989) and all of the different subtypes H1-H14 and N1-N9 of the A virus are perpetuated in these birds especially the migratory waterfowls (Hinshaw *et al.*, 1980, 1986). The adaptation of the A viruses to ducks has resulted in non-pathogenicity of the viruses in these hosts, thus creating a reservoir to ensure their perpetuation. This makes ducks uniquely important in the natural history of influenza viruses and those of avian origin have been implicated in outbreaks of diseases in mammals such as seals (Webster *et al.*, 1978, 1981b), whales (Halvorson *et al.*, 1983), pigs (Scholtissek *et al.*, 1983) and more recently horses (Guo *et al.*, 1992; Shortridge *et al.*, 1995) as well as domestic poultry especially turkeys (Alexander *et al.*, 1993).

The evolution of influenza A viruses also occurs as a result of a variety of factors such as the host range, various virus protein antigens, the evolutionary rates, the receptor binding sites, separate host-specific virus gene pools, interspecies transmission and selection pressures. The HA and NP appear to be the major host range determinants due to the role of the HA in host cell recognition and attachment and the NP's long-term host-specific evolution (Scholtissek *et al.*, 1985; Webster *et al.*, 1992). All the known HA

subtypes occur in the avian hosts but particular HA subtypes occur in mammalian hosts as outlined above. The host species from which the viruses are isolated also determine the receptor specificity of the HA. Thus, human H3 viruses bind exclusively to sialyoligosaccharides terminated by SAα2, 6, Gal while avian, equine and human H1 viruses isolated after 19/7, swine H1 and human H2 viruses specifically bind SAα2, 3, Gal due to Leu-226 in the HA receptor binding site in human H3 and human-like swine H3 viruses instead of Gln-226 in avian, equine and other HA subtype viruses (Roger and Paulson, 1983; Rogers *et al.*, 1983a, b; Yamada *et al.*, 1984; Rogers and D'Souza, 1989). The NA and the virus internal proteins may also play a role in host range restriction (Sugiura and Ueda, 1980; Kristner *et al.*, 1989).

Separate host -specific gene pools exist and interspecies transmission combined with separation of the different host species contribute significantly to evolutionary divergence of influenza A viruses. However, interspecies transmission, if it occurs at all, does not always result in a net flow of genes between existing host-specific virus gene pools in that the reassorted progeny virions with new genes may be unfit to persist because of reduced replication and virus shedding relative to virions with host adapted genes. Although generation of new virus genotypes may occur in a virus gene pool, successful introduction of new genes may be subject to conditions that preclude some genotypes or make them unlikely to appear. However, these genotypes do appear occasionally to cause diseases. Selection pressures impose evolutionary constraints on influenza viruses and each gene may evolve differently because of different selection pressures and constraints. Thus, the surface glycoproteins HA and NA may be subjected to strong selection pressures by

neutralising antibodies produced by the host's immune system while the genes encoding the internal proteins may not but may undergo significant host-specific adaptative evolution (Gorman *et al.*, 1990). The evolutionary rates of homologous genes from host-specific virus strains thus differ and the surface antigens are expected to evolve much more rapidly to produce reassortants with new glycoprotein genes and a selective advantage over the parent viruses previously exposed to the host species. The new viruses would be able to escape the host immune response at least temporarily and if they are sufficiently infectious, they could cause epidemics or pandemics and replace previous strains thus leading to antigenic/genetic shift (Webster and Laver, 1975).

In the historical origin and evolution of influenza viruses, the major events that resulted in a separation or generation of new gene pools leading to lineage divergence from a common ancestor as reviewed by Webster *et al.*, (1992) are summarized in Fig.8. The divergence of the major lineages of the HA and NA subtypes and the split between the NS genes of the A and B alleles probably occurred in the 19th century and the divergence of human influenza B viruses from avian influenza viruses also predates the 20th century. The oldest event among mammalian influenza A viruses seems to be the divergence of Eq/Prague/56 equine-1 (H7N7) viruses the latest estimated date being 1800 A.D. (Gorman *et al.*, 1990). Other events in the 19th century were the origin of the present day H13 gull virus HA, M NS, NP and PA genes (which occurred in two separate reassortments) and the origin of the North American avian virus strains, recent equine-2 strains and the fowl plague virus. Current human viruses originated in the early 20th century just before the 1918 pandemic and the classical swine viruses were probably

Fig.8. Summary of evolution of influenza viruses.

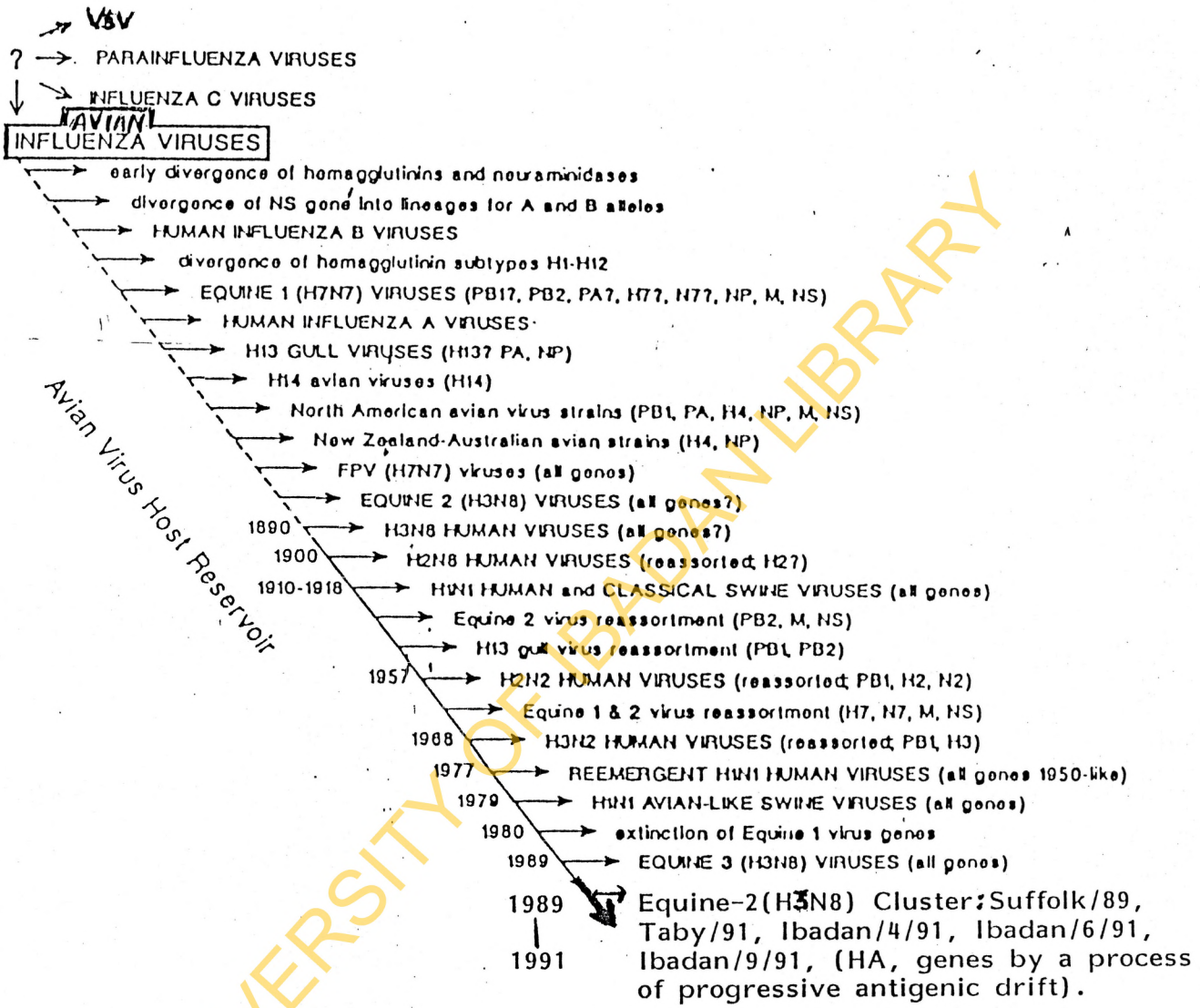


Fig. 8. Summary of the evolution of influenza viruses adapted from Webster et al, [1992]. The vicariant events which led to lineage divergence are roughly in chronological order and are deduced from collective analysis of influenza virus gene phylogenies, antigenic and genetic data as well as historical records. The dashed line indicates an indeterminant time frame. The genes and sub-types involved in evolutionary events are brackets. The EQUINE 3 viruses refer to the new avianlike H3N8 viruses recently isolated in northeastern China (Guo et al, 1992). The 1989/91 cluster viruses are those recently isolated in Northern Europe and Nigeria (See Chapter 4 of text). VSV means vesicular Stomatitis Virus.

derived from human viruses during this pandemic. Reassortment in human and recent equine-2 and H13 gull viruses occurred from the 1920s while the most recent events are the appearance of a new avian- derived H1N1 virus in pigs in Italy in 1977 and H3N8 virus in horses in China in 1989. It seems that these new avian-derived viruses are still evolving. Over the past two and a half centuries, between 10 and 20 human influenza pandemics have swept the world and since the first human virus was isolated in 1933, new subtypes of the A virus have emerged, each of which originated from China. Serological and virological evidence suggests that since 1889, there have been six instances of the introduction of a virus bearing an HA subtype that has been absent from human population for some time thus giving a cyclical appearance of three human HA subtypes with the sequential emergence of H2 in 1889, H3 in 1900, H1 in 1918, H2 again in 1957, H3 again in 1968 and H1 again in 1977 (Webster and Kawaoka, 1994).

In horses, recent work has shown that the H7N7 equine-1 viruses isolated between 1956 and 1963 form a distinct lineage (Gibson *et al.*, 1988; Gorman *et al.*, 1990) while more recent equine-1 viruses have been shown to be reassortants consisting of HA, NA and M genes from Eq/Prague/56-like viruses and internal PB1, PB2, PA, NP and NS genes from equine-2 H3N8 viruses (Adeyefa *et al.*, 1994) thus confirming the genetic shift that had occurred in the latter H7N7 equine viruses. With equine-2 viruses, recent studies have shown multiple evolutionary lineages of the H3 HA (Endo *et al.*, 1992; Binns *et al.*, 1993) in contrast to a single lineage earlier suggested by Kawaoka *et al.*, (1989a). The most recent equine H3 viruses appear to have evolved by a process of antigenic drift away from the original equine-2 prototype virus Eq/Miami/63 (Kawaoka *et al.*, 1989a; Endo *et*

al., 1992; Binns *et al.*, 1993; Adeyefa and McCauley, 1995, Fig.8).

1.7 PATHOGENESIS

1.7.1 Transmission and Pathogenicity

In humans, influenza virus spreads from person to person by the aerosol route (McLean, 1961; Moser *et al.*, 1979) as in animals but in avians transmission is by the oral and aerosol routes (Webster *et al.*, 1992). Most influenza A infections are transmitted via droplets formed during coughing and sneezing but spread by direct contact is also possible. Horses are frequently transported for equestrian events which facilitates easy spread of the viruses. Particles less than 2μm generated during a sneeze or cough are deposited in the lungs to initiate infection (Knight, 1973). The virus replicates throughout the respiratory tract and it is recoverable from both upper respiratory tract within a week and lower respiratory tract up to 10 days in patients naturally or experimentally infected (Kilbourne, 1959; Murphy *et al.*, 1973; Frank *et al.* , 1981). Incubation period is 1-2 days while virus replication peaks at about 48 hours during and after which viruses are shed for up to 6-8 days post infection (Murphy *et al.*, 1973). Persistence of viral antigen in the nasal epithelium has been observed in influenza infection with pulmonary complications (Ebisawa and Kitamoto, 1969) and in mice antigen persistence up to 1 year resulting in protracted alveolitis has been described (Jakab *et al.*, 1983).

Influenza viruses efficiently induce production of interferon to which antiviral properties they are sensitive (Richman *et al*, 1976). Interferon is detectable in the acute

stages of illness in the upper respiratory tract secretions and serum of infected patients about 24 hours from onset of virus shedding and peak titer of interferon is found about a day after virus peak titer (Richman *et al.*, 1976; Green *et al.*, 1982) with a direct correlation between the level of virus multiplication and amount of interferon induced (Murphy *et al.*, 1973; Romagnani, 1992; Schijns *et al.*, 1994).

Both H7N7 and H3N8 subtypes of influenza A viruses have co-circulated in equines (Tumova *et al.*, 1972) although the last known equine-1 outbreaks in horses on the field were in 1977 (Tumova, 1978). The mechanism by which the two subtypes are maintained in equines is not quite clear but it seems likely that the viruses are continuously passed to susceptible animals. It is thus possible for genetic reassortment of the viruses to occur as has been shown by RNA-RNA hybridisation study (Bean, 1984) and nucleotide sequence analyses (Bean *et al.*, 1992; Adeyefa *et al.*, 1994). In horses, the pathogenesis involves the disruption and sometimes complete removal of the protective ciliated epithelium lining the respiratory tract. Secondary bacterial infection resulting from this is common although most horses, if properly rested will recover without antibiotic treatment (Wood and Mumford, 1992). As in man, equine influenza infections are immunizing and are rapidly cleared from the respiratory tract. A current challenge in the field of viral pathogenesis is to understand how virus infection leads to specific tissue damage and disease or death of the host. In influenza infections cleavage of the HA into HA1 and HA2 by cellular proteases at the cleavage site comprising a series of basic amino acids is a prerequisite for the formation of infectious influenza virus (Walker and Kawaoka, 1993). Virus with uncleaved HA is not infectious *in-vivo*. Certain bacteria infecting the lungs

represent a source of proteases capable of proteolytic activation of influenza virus and aggravating viral infections directly or indirectly. Bacteria that have been implicated include Haemophilus *influenzae, Streptococcus pneumoniae, Strept. pyogenes, Pseudomonas aeroginosa, Staphylococcus aureus* and *Aerococcus viridans*. Clearly, fulminating influenza could result from a complex interplay of factors that are determined by characteristics of the bacterial co-infection and the inflammatory responses of the infected host (Nicholson, 1994) and just as bacteria can potentiate viral infections, so viruses can enhance bacterial pathogenicity as in the case of *Neisseria meningitidis* and *H. influenzae* type B.

Influenza A and B viruses cause cytopathic effect (CPE) in infected cells by apoptosis, a mechanism of cellular DNA fragmentation into a 200-base pair ladder and ultrastructural changes which lead to cell death (Hinshaw *et al.*, 1994). CPE due to apoptosis is characterised morphologically by cell shrinkage, hyperchromatic nuclear fragments, nuclear condensation and plasma membrane blebbing. Biochemically, there is chromatin margination and cleavage into nucleosomal oligomers or apoptotic bodies. Induction of apoptosis by influenza A viruses contributes directly to the pathogenesis of infection in the horse.

1.7.2 Clinical Signs

In man, influenza is characterised by a rapid onset with chills, fever, aches and prostration but without prominent respiratory signs initially. The typical uncomplicated

syndrome is a tracheobronchitis with additional involvement of the small airways resulting in bronchiolitis (Little *et al.*, 1976). A dry harsh cough which is rapidly followed by high fever, myalgias, malaise and anorexia, sternal tightness and soreness may accompany the dry cough. The clinical manifestations of influenza in children and adults can be very similar but distinct differences occur in different age groups. Children have very high fever, sometimes with febrile convulsions. There is a high incidence of gastrointestinal manifestations like vomiting and abdominal pain while otitis media, croup and myositis occur more frequently with life threatening febrile reactions in neonates (Meibalane *et al.*, 1977; Kim *et al.*, 1979; Glezen *et al.*, 1980). Mortality is high in young adults below 24 years and older people above 65 years (Barker and Mulloly, 1980; Alling *et al.*, 1981).

In equines, the clinical signs include a dry harsh, hacking and unproductive cough, fever, anorexia, myositis and tracheobronchitis often accompanied by secondary bacterial pneumonia as in man (Bryans, 1975). A high incidence of interstitial myositis has been recorded in infections with H3N8 viruses and other sequelae include purulent pharyngitis, conjunctivitis, sinusitis, chronic laryngitis and bronchopneumonia (Gerber, 1970). The duration and severity of clinical signs depend on the animal's previous exposure to influenza virus by natural infection or vaccination. Horses primarily exposed are more severely affected and develop higher elevated temperatures ranging from 39-42°C for several days while those previously exposed show less elevated temperatures for shorter periods (Powell, 1991). In vaccinated horses, however, the duration and severity of clinical signs are significantly reduced. Recovery is also usually rapid and uneventful, particularly if horses are well rested in the acute stage. In this regard, diagnosis on clinical

grounds alone is very difficult in view of other viral respiratory infections. A salient feature that still distinguishes equine influenza from most other respiratory diseases is the high proportion of "in-contact" animals that show some signs (Wood and Mumford, 1992). Both H7N7 and H3N8 viruses produce a similar disease but H3N8 infections are usually more severe probably because of the greater propensity to invade the lower respiratory system and establish pneumonia (Powell, 1991).

Swine influenza as human influenza is characterised by coughing, fever, nasal discharge, laboured breathing and conjunctivitis (Shope, 1931; 1958). In avian species, the clinical signs are associated with the strain or subtype of viruses involved. Most infections are asymptomatic, some strains cause chronic respiratory infections while a small minority such as A/FPV/Dutch/27 (H7N7) and A/Tern/South Africa/61 (H5N3) A/Turkey/Ontario/7732/66 (H5N9), A/Turkey/England/50-92/91 (H5N1) and A/chicken/ Pennsylvania/1370/83 (H5N2) cause rapidly fatal systemic infections accompanied by central nervous system involvement with death occurring within a week of infection which have led to the destruction of over 17 million birds in Pennsylvania in 1983 (Webster *et al.*, 1986) and 7129 turkeys in England in 1991 (Alexander *et al.*, 1993; Saito *et al.*, 1994). Influenza viruses also infect seals, mink, whales and primates causing diseases and deaths (Geraci *et al.*, 1982; Murphy *et al.*, 1982a; Hinshaw *et al.*, 1984).

1.7.3 Immunity

Immunity to influenza infections is a gradient, depending on its degree, that (i) can suppress infection to make it undetectable, (ii) can modulate infection to prevent

disease or (iii) can reduce the severity of disease (Kilburne, 1987). Influenza infections are immunizing (Wood and Mumford, 1992) and elicit the synthesis of demonstrable serum antibodies to HA, NA, NP and M viral proteins which represent major classes of immunoglobulins G, M and A (IgG, IgM, IgA) (Kilbourne,1987). Immunity to natural influenza infections is long-lived and is sub-type specific. Prior infection with an influenza virus of one serotype bearing highly related internal antigens such as M and NP proteins has no major effect on the occurrence of subsequent infections with viruses of a different subtype. Also, little hetero-subtopic immunity is exhibited by individuals infected with H1N1 and H3N2 viruses in the same season (Potter and Oxford, 1979; Gill and Murphy, 1985). It is thus probable that immunity is primarily mediated by responses to influenza surface glycoproteins. This is consistent with observations on antigenic shifts which involve acquisition of new HA or NA proteins but retention of highly conserved NP and M proteins of the previous strain as seen in the change from H1N1 to H2N2 in man or H7N7 to H3N8 in equines as well as in antigenic drifts which permit influenza A viruses to escape long-term immunity induced by prior infection and involve mutations in the regions of HA and NA glycoproteins that interact with antibody molecules (Noble, 1982).

Immunological response to influenza infections is both humoral and cell-mediated. Studies on the kinetics of serum and secretory antibody response have demonstrated that IgM and IgA responses peak in the second week of infection and IgG two weeks later (Murphy *et al.*, 1982b) with most of the IgA being actively secreted locally. In cell-mediated immunity, antibody response to HA is T-cell dependent. The HAs of different viral subtypes share cross-reactive antigenic determinants recognised by T-helper (T_h) cells

and within a viral subtype the HAs are broadly cross-reactive in T_h-cell recognition (Anders *et al.*, 1984). Haemagglutinin-specific T cells can also recognise determinants different from those seen by anti-HA antibodies (Katz *et al.*, 1985; Sterkers *et al.*, 1985). T_h-cells also play a role in the generation of immune cytotoxic T-lymphocytes (Tc or CTL) response in influenza infection and T_h-cells primed by internal antigens can cooperate in antibody response to HA (Russel and Liew, 1979; Biddison *et al.*, 1981). Reiss and Schulman (1980) have shown that mice infected with influenza viruses develop Tc cells specific for viral antigens prior to appearance of virus-specific antibody-forming cells. These antibody-forming cells are HA specific while the Tc cells of lymph nodes and spleen are cross-reactive among the major histocompatibility (MHC)-compatible cells infected with multiple influenza virus subtypes. Human Fc IgG receptor-bearing null cells and Tc have been shown to mediate a type of lymphocyte toxicity that is antibody dependent (Greenberg *et al.*, 1977) while antibody mediating the "antibody-dependent cell-mediated cytotoxicity" (ADCC) reacts with either HA or NA and it is subtype specific on primary immune response but may exhibit broad heterosubtypic reactivity on secondary infection. This ADCC antibody is demonstrable in the sera of young children after natural influenza infection (Greenberg *et al.*, 1977). CTL response is a central component of the host response to many viruses including influenza viruses. CTL limit viral replication, accelerate clearance of viruses, recognise proteolytic fragments of viral proteins presented at the cell surface by class 1 MHC molecules and protect the hosts against subsequent reinfection (Castrucci *et al*, 1994; Lawson *et al*, 1994). $CD8^+$ MHC class 1 restricted

antiviral CTL are the specific effector-cell subsets which recognise the NP as the dominant viral determinant (Townsend *et al.*, 1986). Thus adoptive transfer of NP-specific CTL clones confer cross-protection from lethal challenge with heterologous influenza A viruses while CTL specific for NS1 and HA epitopes also have protective effects when adoptively transferred to infected individuals (Kuwano *et al.*, 1990; Lawson *et al.*, 1994).

Immunological response in influenza infection is oriented towards the initial priming antigens experienced at first infection and in subsequent infections cross-reactive epitopes shared by the original and the re-infecting viruses will stimulate a predominant secondary antibody response while new epitopes in the re-infecting variant virus will induce a primary response (Francis *et al.*, 1953). This selective amnestic response occurs with both HA and NA antigens and this memory also involves the secretory immune response (Wright *et al.*, 1983) as well as ADCC directed against influenza-virus infected cells (Hashimoto *et al.*, 1983).

Influenza viruses also induce non-specific effects on immune response of the infected individual. The HAs are potent lymphocyte mitogens that specifically simulate B cells (Armstrong *et al.*, 1981; Anders *et al.*, 1984). In mice this mitogen is controlled by MHC (Scalzo and Anders, 1985). On the other hand, intact influenza virus inhibits in-vitro antibody synthesis (Daniels and Marbrook, 1981). However, the significance of these contrasting effects for the total immune response in influenza infection is unknown and calls for elucidation. In humans, infectious virus is more effective in stimulating immune responses including CTL and Th cell responses and immunological memory than

inactivated whole virus or virus sub-units (Webster and Arkonas, 1980). In equines however, studies in experimental ponies have clearly shown that antibody responses stimulated by inactivated whole virus vaccines are short-lived and that multiple doses are required to maintain immunity (Mumford *et al.*, 1983, Chambers, 1992). Natural infections induce a longer lasting immunity for at least one year (Hannant *et al.*, 1988). However, in recent times, it has been observed that vaccinations did not prevent infection, although the severity and duration of disease were reduced (Wood and Mumford, 1992; Lai *et al.*, 1994)). The degree of protection correlates closely with circulating antibody levels in natural and experimental equine influenza infections (Mumford *et al.*, 1990). But the level of antibody required for solid protection against natural infection in horses is not always attained by currently available vaccines (Chambers, 1992; Wood and Mumford, 1992). Furthermore, significant antigenic drift in equine influenza viruses has occurred away from strains contained in currently available vaccines (Binns, unpublished data, Chambers, 1992). The recurrent nature of influenza and the capacity of the virus to change and reinfect is suggestive of transient or imperfect immunity to this unconquered threat to man, animals and birds (Webster *et al.*, 1992).

1.7.4. Diagnosis

The specific diagnosis of influenza depends on the recovery of virus from tissues and secretions, demonstration of viral antigens in infected cells or secretions and measurement of specific immunological responses. Primary isolation can be made from

animal inoculation, chicken embryos or cell cultures. The original recovery of human influenza virus was in the ferret which still has a place in influenza research in providing strain-specific antisera and in testing antiviral agents. Isolations are also possible in mice (Francis and McGill, 1937) and hamsters (Taylor, 1940). The chicken embryo was first used for propagating influenza virus in 1935 (Smith, 1935) and for isolating it in 1940 (Burnet, 1940). It has remained the standard host for isolation and growth of the virus ever since. The amniotic and chorio-allantoic membrane endodermal cells of the developing embryo are very susceptible to the virus and can yield up to $10^{10}EID_{50}$ of infective influenza A virus per 1ml of allantoic fluid. Allantoic sac inoculation is frequently used in 10-12 day old chicken embryos usually employed in virus isolation but 13-15 day old embryos can be used for amniotic inoculation (French and Dineen, 1958) especially for influenza C viruses. These are incubated at 33-37°C for 26-48 hours or more depending on the host species, type or subtype of the virus being isolated (WHO handbook, 1982) before the virus laden allantoic fluid is harvested after adequately chilling the eggs. A variety of primary cell cultures on the other hand will support replication and plaguing of influenza viruses but the use of primary cell cultures has now been superseded by the use of a continuously propagable cell lines such as MDCK (Davies *et al.*, 1978), MDBK, BHK, RS2, BSC40, LT, VERO and a host of others which are also very efficient in detecting the virus.

Serological tests used in diagnosis and identification of influenza viruses include haemagglutination-inhibition (HI), virus neutralisation (VN), complement fixation (CFT), neuraminidase-inhibition and activity (NAI) assay, single radial haemolysis, single and

double radial immunodiffusion tests, enzyme-linked immunosorbent assays (ELISA) as well as monoclonal antibody profiling through monoclonal antibody panels. More advanced molecular biology techniques of oligopeptide mapping, RNAse T1 oligonucleotide fingerprinting and multiplex RT/PCR based on the conserved terminal sequences of the virus genes (Adeyefa *et al.*, 1994) have been developed for use in molecular epidemiology and virus surveillance as well as rapid diagnosis and analysis of influenza virus genes for changes in virus genotypes. Rapid diagnostic kits have also been developed for use in direct and indirect immunofluorescence of cells and ELISA. They are commercially available and include Imagen (Baxter Bartels Microscan) and Directigen FLU A (Dominguez *et al.*, 1993). Field diagnosis is however based on clinical signs and epidemiology of influenza infections.

1.7.5 Treatment, Prevention and Control.

The use of antiviral drugs has not yet found a practical application in the treatment of equine influenza. Complete rest is very essential for the recovery of affected horses and this should be accomplished by resting a horse for a week for each day of elevated temperature. Any form of exercise should be avoided until a week after most of the clinical signs have abated, although occasional coughing may be observed for several weeks particularly after mild exercises. The clinical signs of equine-2 infection are more pronounced than those of equine-1 infection as a result of lower respiratory system involvement. Secondary bacterial infection is thus very common. Antibiotics should be given for 10-15 days to horses that develop secondary bacterial infections. The

environment of affected horses in particular and all horses in general should be kept dust-free as much as possible to minimise irritation of the respiratory tract. Good nursing care and improved nutrition are essential for sick horses.

In man, antiviral drugs such as amantadine hydrochloride and its analogue, rimantadine are used in the prophylactic and therapeutic treatment of influenza A infections but have no antiviral properties against influenza B and C viruses (Nicholson, 1994). The use of amantadine for horses has now been proposed although amantadine-resistant phenotype is suspected to occur in the horse (T. Chambers, personal communication). Isoprinosine on the other hand is effective against influenza B and C viruses *in-vivo* (Muldoon *et al.*, 1973). The antiviral activity of these drugs is exerted after adsorption of the virus to the cells but before primary transcription occurs (Richman *et al.*, 1986).

In man and animals prevention is achieved by routine vaccination. In equines, inactivated vaccines containing both subtypes of influenza A virus antigens are used extensively either alone or in combination with tetanus, herpesvirus and viral encephalitides vaccines (Powell, 1991). Influenza vaccines contain either whole or subunit viral antigens combined with an adjuvant and are administered by intramuscular injections. The schedule of vaccination requires two primary injections given 2-4 weeks apart followed by an annual booster. Current vaccines contain the prototype equine-1 virus strain (A/Eq/Prague/1/56, (H7N7) and two equine-2 strains, prototype A/Eq/Miami/63, (H3N8) and either A/Eq/Fontainebleu/79 or A/Eq/Kentucky/81, H3N8 strains. But a poor duration of immunity has been observed with currently available vaccines generally lasting

3-4 months (Powell, 1991, Mumford *et al.*, 1990, Chambers, 1992). This is in contrast to human vaccines in which their efficacy has considerably reduced influenza associated hospitalisation particularly in elderly individuals (Foster *et al.*, 1993). Although the degree of protection against clinical disease correlates closely with circulating antibody levels, the antibody level required to induce solid protection against infection is not always attained by these currently available vaccines other than for brief periods (Wood and Mumford, 1992). Moreover, it has recently been observed that significant antigenic drift in equine influenza virus has occurred away from strains contained in the currently used vaccines which now require upgrading (Kawaoka *et al.*, 1989a; Endo *et al.*, 1992; Binns *et al.*, 1993).

A new subtype H3N8 influenza virus, A/Eq/Jilin/1/89 recently isolated from clinically affected horses in China has been shown to be antigenically and genetically different from previously circulating equine-2 influenza viruses (Guo *et al.*, 1992). Antigenic analyses of Eq/Jilin/1/89 virus showed only low cross-reactivity with chicken sera against Eq/Miami/63 or Eq/Tennessee/5/86 viruses and much higher cross-reactivity with chicken sera against the avian H3N2 virus strains (Guo *et al.*, 1992). This absence of a high level of antigenic cross-reactivity raises the possibility that the currently available equine influenza vaccines may not protect horses against outbreaks of Eq/Jilin/1/89 virus. If this were the case future occurrences of Eq/Jilin virus infection in horses would necessitate the development of new equine influenza vaccines that would contain Eq/Jilin virus antigens in addition to the currently contained equine-1 and 2 antigens (Chambers,

1992). There has been considerable interest in developing alternative live vaccines such as temperature sensitive and recombinant products that could mimic the response of solid immunity to natural infection more effectively for at least a year and promote a humoral as well as cellular response (Holmes *et al.*, 1988; Dale *et al.*, 1988). An inactivated subunit vaccine containing HA and NA surface antigens integrated into an immunostimulating complex (ISCOM) system has been developed in Scandinavia (Sundquist *et al.*, 1988) which consists of a matrix composed of a glycoside, Quill A, extracted from the bark of a South American tree. The matrix holds the HA and NA antigens and acts as an effective adjuvant.

In humans, inactivated influenza A and B virus vaccines are used for parenteral administration and usually contain currently circulating strains of the viruses. Currently available vaccines include whole virus, split-product virus and subunit vaccines. The whole virus vaccines contain intact, formalin-treated virus, the split-product vaccines contain purified formalin-treated virus disrupted with chemicals such as sodium dodecyl sulphate and N- Lauryl Sarkosyl that solubilise the lipid-containing viral envelope while the subunit vaccines contain isolated HA and NA surface protein antigens. Generally, antibody responses to the split-product and subunit vaccines are similar but previous exposure to an influenza A virus within the same subtype appears to be an important determinant of serum antibody responses to the split-product and whole virus vaccines (Mostow *et al.*, 1970). As in equines, the degree of protection correlates with the level of circulating antibodies and parenteral vaccination consistently induces resistance to illness and, to a lesser extent, infection with influenza A and B viruses (Sears *et al.*, 1988). However,

polyvalent vaccines containing antigens unrelated to the subtype present in an epidemic virus may not induce resistance to natural challenge (Mostow *et al.*, 1970). Annual vaccination is currently recommended for high risk groups of people who have chronic conditions such as diabetes, cardiac, bronchopulmonary, renal and other metabolic diseases, regardless of their age. Individuals over 65 years old are also to be vaccinated annually while pregnant women should be vaccinated for several years following an influenza epidemic. Recombinant live vaccines have not been licensed for use in humans but experimental live vaccines have been developed and evaluated for their prophylactic effects and efficacy in man (Murphy *et al.*, 1982b).

1.7.6. Influenza in Nigeria

There is paucity of information on the occurrence, clinical manifestation, isolation, characterisation and epidemiology of influenza in various host species in Nigeria in particular and generally in tropical Africa generally as earlier stated. David-West and Cooke (1974) reported an influenza outbreak in humans from whom a virus, A/Nigeria/1/74 (H3N2) very similar to A/Port Chalmers/73 (H3N2) was isolated. Outbreaks were also reported along the West African coast in Gambia and Senegal (McGregor *et al.*, 1979) about this time as a result of Influenza epidemics from 1968 virus that was sweeping the world. Since then, to my knowledge, influenza has not been officially reported in man, animals and birds until recently when HI antibodies to some human influenza virus strains were demonstrated in the sera of horses, pigs and chickens (Olaleye *et al.*, 1990, Adeniji *et al.*, 1993). Durojaiye and Denya (personal

communication) in early 1982 found antibodies to equine-1 and 2 viruses in the sera collected from some 129 horses in the late 1970s at the prevalent rates of 50.3 % and 15.5% respectively. These reports and observation suggest previous circulation of influenza A viruses in various host species in Nigeria, although no viruses have been isolated from these hosts so far. Studies were thus designed to isolate and characterise influenza A viruses from equines in Nigeria as outlined in the introduction.

CHAPTER 2

ISOLATION, IDENTIFICATION, ANTIGENIC AND POLYPEPTIDE ANALYSES OF EQUINE INFLUENZA VIRUSES

2.1 INTRODUCTION

Equine influenza has been recognised as the cause of epidemic outbreaks of respiratory disease among horses for centuries and has been associated with human influenza epidemics (Hirsch, 1883; Kilbourne, 1987). In recent years, it has become increasingly implicated as a significant infection of horses in many areas of the world. All outbreaks from which a virus has been recently isolated have been caused by antigenic variants of classical equine-2 (H3N8) influenza virus apart from the outbreak that occurred in northeast China in 1989 with high morbidity and mortality that was caused by a novel H3N8 virus (A/Eq/Jilin/1/89) of avian origin (Guo *et al.* 1992, Shortridge *et al.*, 1995). The incidence of these outbreaks as outlined in chapter 1 appears to have also increased with the increase in intercontinental movement of horses for equestrian events and breeding. This can invariably enhance the emergence of new virulent strains of viruses which can in turn cause epidemics and/or pandemics.

Compared with some other areas of the world, for example, Europe, North and South America and the Far East, the incidence of equine influenza in Africa particularly West and East Africa, is very low considering available records. There is paucity of information on equine influenza in tropical Africa particularly Nigeria despite regular movement of horses into this area. Horses are sometimes imported into this country from

Europe and South America particularly for polo games and from neighbouring countries into the north of the country for local racing. Humans and equines live in very close association particularly during equestrian events and there has been notable increases in acute respiratory tract infections in both species in recent times most of which are simply diagnosed as common cold. In view of the potentials of influenza virus for rapid global spread and for causing acute respiratory disease in equines, the importation of horses into the country and all year round equestrian events that take place annually in the north and the southwest, it is conceivable that equine influenza viruses could be circulating among equines in Nigeria. In the light of the paucity of information on any influenza viruses circulating in the country since the isolation of a virus from humans in 1974 (David-West and Cooke, 1974), studies were designed for extensive nationwide equine influenza virus surveillance, isolation and characterisation of viruses from equines to document and provide this hitherto unavailable information in this region. The results of virus isolation, antigenic and virus polypeptide analyses are reported in this chapter.

2.2 MATERIALS AND METHODS

2.2.1 Sample collection

From January 1989 to January 1995, naso-pharyngeal swabs were collected from symptomatic and asymptomatic animals into sterile sample bottles containing virus transport medium (Eagle's medium without serum) and transported to the laboratory on ice for virus isolation. Samples were obtained from (a) horses used for polo games in Ibadan, Lagos, Kaduna, Kano, Jos, Minna, Sokoto, Yola and Port Harcourt polo club

stables. (b) horses used for local racing in Kano, Maiduguri, Sokoto and Kaduna; (c) Nigerian Police Mounted Troop horses based in Lagos (Obalende and Ikeja), Ilorin, Kaduna, Kano, Jos, Maiduguri, Biu and Abuja (d) Military horses based in Nigerian Defence Academy in Kaduna and the Saddle Unit in Ibadan (e) horses and donkeys in University of Ibadan Veterinary Teaching Hospital and Zoological Garden (f) privately owned horses used for ceremonial purposes in some southwestern and northeastern States (g) horses at the horse market at Ibagwa Akwa and (h) horses slaughtered for human consumption in Oboloafor both near Nsukka in the East (i) donkeys from the donkey markets in Sokoto, Kano and Ilorin (j) horses and donkeys used by Fulani herdsmen in the rural areas of Oyo North and Bornu State. Particular attention was paid to animals showing signs of respiratory diseases including oculonasal discharges. Animals of all age groups were sampled. Tissues from the upper and lower respiratory tracts from five animals were also collected at post mortem for swabs and homogenates. A total of 1,071 swabs and 5 tissue homogenates were obtained and stored at -70°C until required. Fig.9 shows the geographical areas of sampling in the country while table 1a shows the date and number of samples collected. One hundred and twenty swab samples that were collected between January 1989 and December 1991 and 160 samples collected between January and March 1993 were taken in a frozen state to the Pirbright Laboratory of the Institute for Animal Health in the United Kingdom where some parts of the studies were carried out. Samples were obtained more than once from some of the horses brought to the Ibadan and Lagos annual polo tournaments as well as the resident horses in both places.

Table 1a: Date, location and number of swab samples collected between January 1989 and January 1995.

Date of Collection	Location	No. of Samples
13/1/89-24/1/95	Ibadan Polo Club	211
" "	University of Ibadan, VTH & Zoo	10
" "	Army Sadde Unit, Ibadan	8
19/5/90-24/1/91	Lagos Mounted Troop	65
16/1/90-12/2/91		
27/1/93-30/3/95	Lagos Polo Club	270
30/4/91	Kaduna Mounted Troop	23
17/1/90-30/4/91	Kaduna Polo Club	33
28/4/91	Nig. Defence Academy, Kaduna	25
15/1/90-20/1/91	Kano Polo Club	13
24/5/91	Kano Mounted Troop	16
25/5/91	Kano Race Course	22
16/1/90-14/1/91	Jos Polo Club	18
2/6/91	Jos Mounted Troop	27
30/5/91	Maiduguri Mounted Troop	68
28/5/91	Maiduguri Race Course	51
6/6/91	Biu Mounted Troop	23
12/1/91	Sokoto Polo Club	9
10/7/93-30/9/93	Sokoto Horse	49
2/2/89	Ilorin Mounted Troop	37
13/10/93	Ilorin Horse/Donkey Markets	6
27/12/94	Ibagwa Aka Horse Market	3
28/12/94	Oboloafor Slaughter House	5
3/3/91-10/11/94	Private Horses Oyo State	6
15/5/91-5/6/91	Private Horse Borno State	9
21 - 24/4/90	Minna Polo Club	10
28/1/90	Yola Polo Club	4
29/1/91	PortHarcourt Polo Club	4
26/2/93-10/3/93	Abuja Mounted Troop	46
Jan. 199-Dec. 1994	Post Mortem Lung Homogenates at UI	5
	TOTAL	1,076

Fig. 9: Map of Nigeria showing the geographical areas of sample collection.

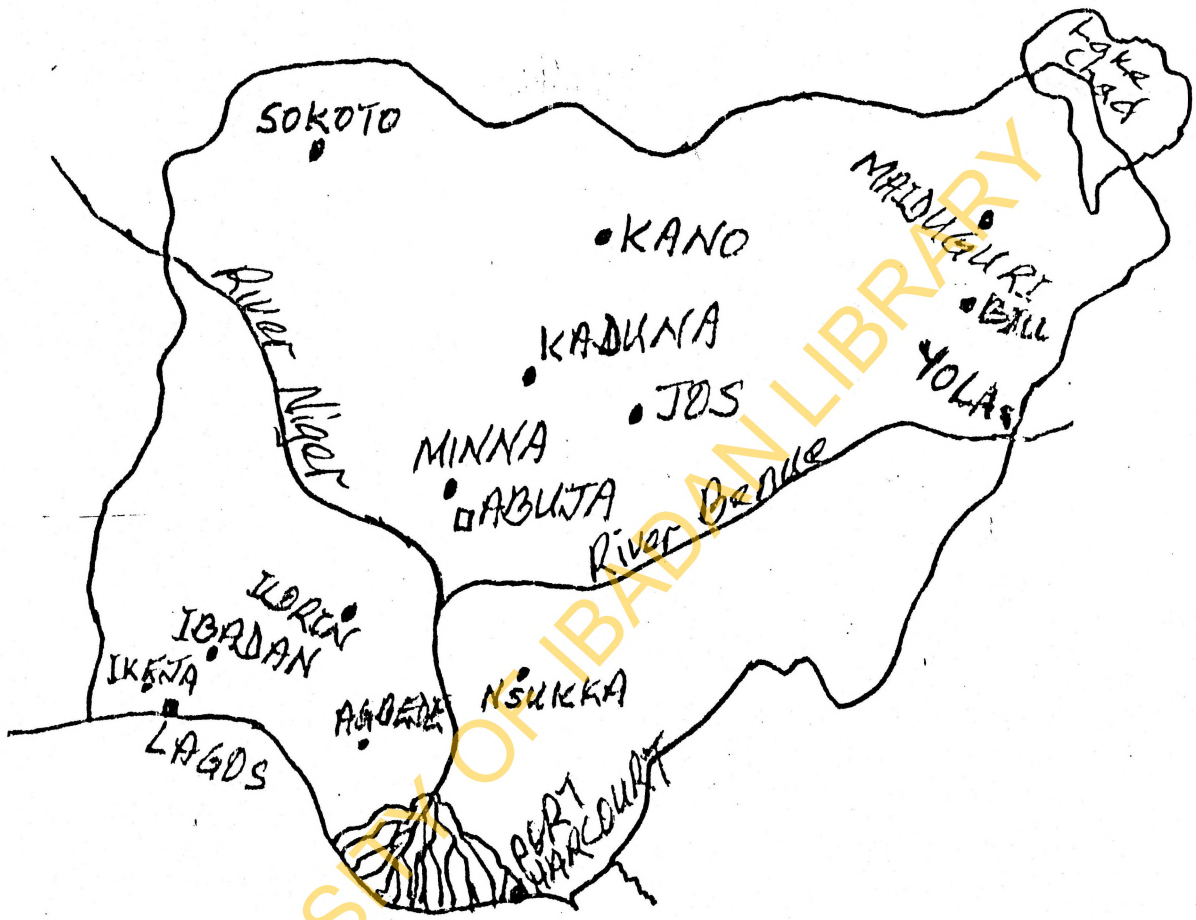


Fig.9: Map of Nigeria showing areas of sampling and the geographycal spread of equine influenza virus activity.

2.2.2 Virus isolation in embryonated hen eggs

The frozen samples were thawed and the swabs squeezed out. The clinical samples were diluted 1:10 in sterile phosphate buffered saline (PBS) containing 200μg/ml penicillin, 100μg/ml streptomycin and 2μg/ml fungizone. One hundred microliters (μl) of each diluted sample were inoculated into three 10-11 day old embryonated (fertile) hen eggs per sample via the allantoic cavity after the eggs had been thoroughly disinfected with methylated spirit. The eggs were incubated at 35°C for 48-72 hours (hr) and chilled at 4°C overnight or at -20°C for 2 hr after which the allantoic fluid (AF) was harvested following the death of the embryos. The AF from 3 eggs per sample was pooled into a 50ml centrifuge tube and clarified by centrifugation at 2500 revolutions per minute (rpm) for 10-15 min at 4°C in a bench top Central 4R general purpose centrifuge. Clarified AF was then tested for haemagglutination (HA) of 1% chicken or guinea-pig red blood cells (rbc) in 96-well U-bottom microtiter plates. Positive samples that haemagglutinated the rbc were marked master stocks while negative samples were repassaged 5-6 times more before being discarded. Each master stock was diluted 1:10 in sterile PBS containing antibiotics and inoculated into 30 (a tray) of 10-11 day old fertile hen eggs and incubated as above. AF was clarified and tested for HA of 1% rbc. Positive samples were marked sub-master stocks. A second egg passage was made from these to obtain the working stocks which were then used for subsequent studies. All the stocks were stored at -20°C for a few days for immediate use or at -70°C until required.

2.2.3 Virus isolation in Mardin Darby canine kidney (MDCK) monolayer cells.

Two hundred μl of diluted clinical samples were inoculated into MDCK monolayer cells in 25ml tissue culture flasks (Falcon) as described in the United States Department of Human and Health Services/Center for Disease Control (DH/CDC) handbook edited by Kendal *et al.* (1982). The composition of the growth medium was 500ml Eagle's minimum essential medium (MEM), 0.132% sodium bicarbonate, 0.14% bovine serum albumin (BSA), 200μg/ml penicillin, 100μg/ml streptomycin, 2μg/ml fungizone and 0.00025% TPCK-trypsin (Sigma). The medium was warmed to at about 35°C before use.

Tissue culture medium was decanted from the cells which were washed twice with MEM without serum, 200μl of each clinical sample inoculated into the flasks and incubated at room temperature of 22-25°C for 15-20 min with rocking every 5 min. After this 4ml of virus growth medium with TPCK-trypsin was dispensed into each flask which was loosely capped and incubated at 34°C in a 5% CO_2 humidified incubator. The caps were tightened on the second day and the cells were checked for cytopathic effect (CPE) from the third day of incubation until the seventh day. The tissue culture supernatant was harvested from each flask and tested for HA with 1% rbc from the fourth day of incubation or from the second day of showing CPE. Positive samples were marked master stocks and all the culture supernatants were re-passaged thrice more. Since adenovirus, paramyxovirus and alphavirus also agglutinate rbc and could sometimes be isolated in fertile hen eggs and tissue culture cells, samples that showed CPE but failed to haemagglutinate rbc were then inoculated into HeLa cells to check if any of these other

viruses were present in the clinical samples tested. All the monolayer cells were stained with methylene blue and examined with the inverted microscope.

2.2.4. Haemagglutination test (HA)

One percent suspension of chicken or guinea-pig rbc was prepared from 10% heparinised whole blood washed thrice in normal saline or PBS. Haemagglutination test was performed as described by Barret and Inglis (1985). Briefly, serial two fold dilutions of clarified AF were performed using 25μl volumes of PBS or saline in sterile microtiter plates (Sterilin) starting with 50μl of undiluted working stock AF followed by addition of 25μl of 1% rbc suspension to each well using a multichannel micropipet. The plates were gently agitated to mix the contents and were left at room temperature for 45-60 min for the rbc to settle after which the end point was determined against a white background. The results were expressed as haemagglutination units (HAU).

2.2.5. Antigenic analysis

Clarified AF and tissue culture medium of samples that were positive by HA were reacted in antigenic cross-reactivity tests with monospecific antisera against Paramyxoviruses, Rhinoviruses, Alphaviruses, Adenoviruses, Herpesviruses, African horse sickness viruses and Equine arteritis virus antigens and known polyclonal antisera to the nucleoprotein (NP) of influenza virus types A, B and C to determine the type of viruses that were isolated using agar gel precipitation, double immunodiffusion, virus neutralisation, haemagglutination inhibition and enzyme -linked immunosorbent assay

(ELISA). Antisera against these viruses were from Pirbright Laboratory repository. Following the results of these tests which showed that the virus isolates were influenza A viruses, polyclonal antisera and monoclonal antibodies (Mabs) raised against the HA and NA of influenza A virus subtypes H1-H14 and N1-N9 were used in haemagglutination inhibition (HI) and neuraminidase inhibition (NAI) assays to determine the subtypes of the viruses that were isolated. Mabs and antisera were kindly provided by Mr. Allan Douglas of WHO Influenza Collaborating Center, National Institute for Medical Research, Mill Hill, London, U.K., Dr. R.G. Webster, St. Jude's Children Research Hospital, North Lauderdale, Memphis, Tennessee, USA and Dr. John W. McCauley, Institute for Animal Health, Pirbright Laboratory, U.K.

2.2.5.1. Virus subtyping with haemagglutination inhibition test (HI).

HI was performed in microtiter plates as described in the DH/CDC hand book with Mabs and antisera. All the antisera were treated with potassium-M-periodate to preclude non-specific inhibition. Fifty μl of saline or PBS were pipetted into first row wells of plates and 25μl into rows 2 to 12 wells followed by 5μl of Mabs or 1:10 dilution of treated antisera into first row well and serial dilutions across in 25μl volumes from rows 1 to 11 while row 12 served as antigen control wells. Twenty five μl of clarified AF were then added to all the wells followed by 25μl of 1% rbc. The plates were gently agitated and left at room temperature for 30-60 min after which they were checked for haemagglutination inhibition against a white background and the end point determined. The antisera used were raised in rabbits against H7N7 and H3N8 equine viruses:

1) A/Eq/Miami/63 (H3N8)

2) A/Eq/Fontaineblue/79 (H3N8)

3) A/Eq/Switzerland/76 (H3N8)

4) A/Eq/Newmarket/76 (H3N8)

5) A/Eq/Prague/56 (H7N7)

6) A/Eq/Miami/63 H3N8 HA monospecific

7) Goat monospecific antiserum to equine-2 HA

Antisera 1-5 were from Mr. A. Douglas while 6-7 were from Dr. Webster.

The Mabs used were anti equine H3 HA :

1) A/Eq/Miami/63 clones H1, H3, H4, H6.

2) A/Eq/Fountaineblue/79 clones H1, H2.

3) A/Eq/Tennessee/5/86 clones 2/1, 4/1, 14/1, 22/1.

4) A/Eq/Miami/63 clones EQ1, EQ5, EQ6, EQ7

5) A/Eq/Kentucky/81 clones 1/1, 2/1, 3/1, 4/1.

6) A/Eq/Iowa/86 clone 1/1, 1/2.

Mabs 1-2 were from Mr. Douglas while 2-6 were from Dr. Webster.

HI titers were expressed as the reciprocals of the highest dilution inhibiting 4 HA units of the virus.

2.2.5.2. Neuraminidase inhibition assays

Both the spot test and the full inhibition assays were carried out as described in the Diagnostic Virology Laboratory procedure of the National Veterinary Services

Laboratories, Ames, Iowa and the DH/CDC handbook. The following reagents were prepared:

1) 0.4M PBS pH 5.9 : 5.678gm disodium hydrogen orthophosphate dihydrate ($Na_2HPO_4.2H_2O$ -Sorensen's salt) was dissolved in 100ml sterile distilled (sd) water as solution A pH 3.94 while 5.52gm of sodium dihydrogen orthophosphate ($Na2HPO_4.2H_2O$) was also dissolved in 100ml sd water as solution B pH 9.46 and 19ml of solution A was added to 81ml of solution B. The pH of the resultant solution was adjusted to 5.9 with drops of solution B.

2) Periodate reagent : 4.28gm of sodium-M-periodate (Sigma) was stirred in 38ml sd water in a beaker to dissolve and transferred into a 100ml bottle and 62ml of conc. orthophosphoric acid was added in a chemical hood and the two solutions mixed. The bottle was wrapped with aluminium foil and stored at room temperature in a dark cupboard.

3) 50% arsenite reagent : 25gm of sodium arsenite (Sigma) was stirred was in a beaker covered with aluminium foil to dissolve. The solution was transferred into a 100ml bottle and 0.75ml of conc sulphuric acid was added in a chemical hood and stored at room temperature.

4) 0.6% thiobarbituric acid : 0.6 gm of thiobarbituric acid was stirred in 100ml sd water on a hot plate covered with aluminium foil to dissolve. After cooling the solution was transferred into a 100ml bottle and stored at room temperature.

5) Fetuin : 100mg fetuin (Sigma) was stirred in 2ml sd water to dissolve after which 2ml of 0.4M PBS pH 5.9 was added to give a final concentration of 25mg/ml.

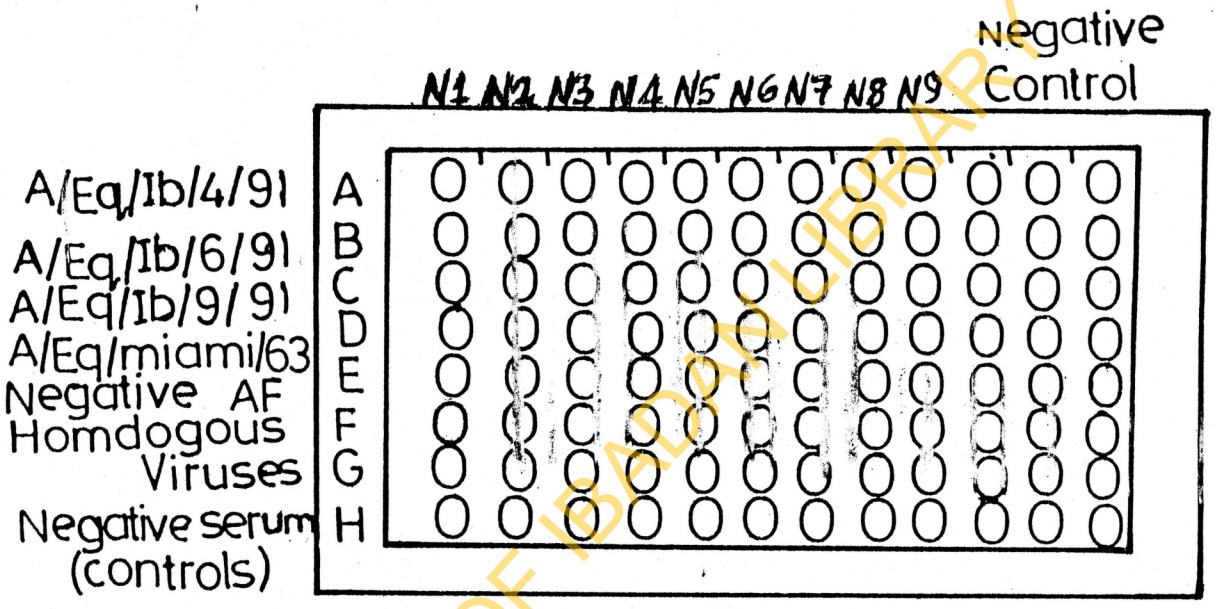
(A) Neuraminidase spot test

The test was performed in a 96-well microtiter plate. A 1:12 dilution of infectious allantoic fluid (AF) of the new virus isolates and homologous viruses to the antisera used was prepared. The plate was marked as indicated in Fig. 10.

Fluid harvested from an uninfected fertile hen egg served as negative AF and serum from a pathogen-free guinea-pig from the laboratory animal house at Pirbright Laboratory served as negative serum. The homologous viruses were grown in 10-11 day old embryonated hen eggs as were the new virus isolates. All the antisera and the negative control serum used were pre-treated with potassium-M-periodate to preclude non-specific inhibition.

Twenty five μl of 1:12 dilution of infectious AF of the new virus isolates and homologous viruses were pipetted into each of 9 wells as marked for that virus and 25μl of 0.4M PBS pH 5.9 were pipetted into the negative control wells. Twenty five μl of 1:10 dilution of anti-NA antisera against influenza virus subtypes N1-N9 and the negative control serum were then added to the corresponding wells. The plate was shaken for 1 min on an orbital shaker to mix the contents covered to prevent evaporation and incubated at room temperature for 1 hr. Twenty five μl of fetuin (25mg/ml) were added, the plate was covered, shaken and incubated at 37°C for 3 hr. After incubation, 25μl of periodate reagent were added, the plate was shaken and incubated at room temperature for 20 min covered. This was followed by adding 25μl of 50% sodium arsenite to each well whereby a dark brown iodine colour developed. The plate was shaken until the colour faded to very light brown and 100μl of 0.6% thiobarbituric acid were added. The plate was then

Fig. 10 Diagram of neuraminidase spot test microtiter plate.



covered with a transparent plastic adhesive tape which was perforated with a 19 gauge needle over each well to allow for expansion in the next step. The plate was floated in a 56°C water bath for 30 min after which it was blotted dry and inverted to remove condensation from its inner surface. The test results were read as follows:-

(i) pink colour indicates no inhibition and the NA is not the designated subtype .

(ii) no pink colour, greatly reduced colour or dirty white colour comparable to control wells containing known antigen and the negative control serum wells indicates inhibition. This is the neuraminidase subtype of the test virus isolate.

The anti-NA to subtypes N1-N9 used and the homologous viruses were from Dr. McCauley's repository while the anti-Miami/63 serum and homologous virus were from Mr. Douglas. The homologous viruses used were:

(1) SD17: A/FPV/Rostock/3453 (H7N1)

(2) X31: A/PR8/Aichi/2/68 re-assortant with PR8 internal genes (H3N2)

(3) LANGHAM: A/FPV/England/1/63 (H7N3)

(4) TYK-ONT: A/Turkey/Ontario/0118/68 (H8N4)

(5) DK-ALB: A/Duck/Alberta/60/76 (H2N5)

(6) GULL-MD: A/Gull/Maryland/704/77 (H13N6)

(7) SD1: Recombinant A/FPV/Rostock/35 x A/FPV/Dobson/27 (H7N7)

(8) DK-IRE: A/Duck/Ireland/83 (H5N8)

(9) DK-MEM: A/Duck/Memphis/546/74 (H11N9)

(10) EQ-MIA: A/Equine/Miami/63 (H3N8)

(11) EQ-NMK: A/Equine/Newmarket/76 (H3N8)

The result of NA subtype from the spot test was confirmed by measuring the anti-neuraminidase titer of a reference antiserum to A/Eq/Newmarket/76 and A/Duck/Memphis/546/74 in the full NAI test.

(B) Full NAI assays

The full test was performed as described in the DH/CDC handbook using 12 x 75mm polypropylene tubes with caps (Falcon 2063).

(i) Determination of total sialic acid:

One milligram per millimetre or 1μg/μl of N-acetyl neuraminic acid (NANA) from Sigma was prepared in duplicate by adding 5μl of fetuin solution (50μg protein/ml) to 100μl sd water. Sialic acid standards were prepared in duplicates by adding 10μl (10μg) and 20μl (20μg) aliquots of NANA (sialic acid) standard 1μg/ml to 100μl of sd water. Reagent controls containing 100μl of sd water were prepared in duplicates. To each tube was added 100μl of 50mM sulphuric acid and the tubes were reacted at 80°C for 1 hr in a prewarmed water bath. After cooling in a 37°C water bath, free sialic acid was measured for each sample by the chemical analysis method described as follows: 150μl of 0.4M PBS pH 5.9 was pipetted into a Falcon tube as reagent control. Four tubes were marked free sialic acid and 100μl of 0.4M PBS pH 5.9 was pipetted into each. Ten μl of sialic acid (10μg) was pipetted into each of 2 tubes and 20μl (20μg) into each of the other 2 tubes. To each tube of 11 tubes from above was added 250μl of periodate reagent, the reactants were mixed and incubated in a 37°C water bath for 20 min with the tubes covered. To each tube was then added 300μl of sodium arsenite, reactants were mixed until a brown iodine colour developed. The tubes were then shaken for 20-30 min until the colour faded

to very light brown. Extra drops of arsenite reagent were added if the colour persisted. Two ml of thiobarbituric acid were then added to each tube, mixed and kept for 8 min in a boiling water bath. The tubes were cooled on ice for 5-10 min and the pink chromogen was extracted into an organic solvent by adding to each tube 4ml of a mixture of butan-1-ol/5% of 10N HCl followed by vigorous vortexing and centrifugation at 4,000 rpm for 5 min. The optical density (OD) of the top organic phase was read at A_{549} nm in a Shimadzu double beam UV spectrophotometer with the white light using the reagent control sample as blank.

(ii) Standardisation of virus antigens:

Two hundred μl serial dilutions of infectious AF of the new virus isolates made in eggs and MDCK cells as well as homologous viruses were prepared in 96-well microtiter plates from 1:2 to 1:128. Seven Falcon tubes were marked 1:2 to 1:128 for each virus antigen and 50μl of 0.4M PBS pH 5.9 was pipetted into the bottom of each tube. Fifty μl of fetuin (25mg/ml) were added to each tube and mixed. A reagent control tube containing 100μl of 0.4M PBS pH 5.9 was also prepared. All the tubes were covered and incubated at 37°C for 18 hr. Free sialic acid was then measured by the chemical analysis method as above.

For each antigen the virus dilutions tested were plotted in a graph on the horizontal axis against the OD values on the vertical axis and the points were joined by a line of best fit for each virus. A position was marked on the vertical axis corresponding to twice the OD value obtained for the standard containing 20μg of sialic acid and a horizontal line was drawn across the graph from this point. From the point of intersection of the two lines

a vertical line was drawn to the horizontal axis. The value at the point where this line intercepts the horizontal axis was taken as the virus dilution to be used in the full NAI assay.

(iii) Measurement of neuraminidase inhibition:

In marked Falcon tubes dilutions of anti-NA antisera in 0.4M PBS pH 5.9 were prepared. Dilutions of 1:10, 1:32, 1:100, 1:320, 1:1000, 1:3200, 1:6400, 1:10,000 and 1:12,800 were used. Starting with the highest dilution for each series, 50μl of antiserum were pipetted into the bottom of appropriately marked tubes. A negative serum control was also prepared for each series. Duplicate tubes containing 50μl of 0.4M PBS pH 5.9 were similarly prepared for each virus to serve as antigen controls and two extra tubes containing 150μl of the PBS served as reagent controls. To all appropriate tubes except reagent controls were added 50μl of virus dilutions as earlier determined. All tubes were mixed and incubated for 1 hr at room temperature in an antigen:antibody reaction. To each tube was then added 50μl of fetuin substrate at 25mg/ml. A substrate control was then prepared by adding 50μl fetuin to 100μl of PBS. The tubes were covered and incubated at 37°C for 18 hr after which the free sialic acid was measured by the chemical analysis method as above.

NAI titers were expressed as the reciprocals of the dilution of antiserum inhibiting 50% of neuraminidase activity in the assay.

2.2.6 Virus purification.

For each new virus isolate, three trays (90 eggs) of 10-11 day old fertile hen eggs were each inoculated with 100μl of 1:10 dilutions of the infectious AF working stock via the allantoic cavity after disinfection. The eggs were incubated at 35-37°C for 72 hr and the AF was harvested into 1 litre conical flasks, clarified in 1 litre plastic bottles in MSE Coolspin centrifuge (Fisons) and tested for HA of chicken rbc as above. The clarified AF were then decanted into 200ml plastic Beckman bottles, sealed and centrifuged at 18,500 rpm for 90 min at 5°C using a Beckman type 19 rotor in a Beckman L8-70 ultracentrifuge to pellet the virus. The pellets obtained were soaked in 1.5ml Ca^{++} and Mg^{++} free sterile PBS in each bottle overnight on ice at 4°C. The pellets were resuspended by sonication at 30 micron, pooled for each virus and carefully layered onto a sucrose gradient made up with 5ml of 60% sucrose w/v in sterile PBS plus 5ml of 30% sucrose w/v mixed with a vacuum pump on top of which was added 5ml of 15% sucrose in 5/8 x 4 inch ultraclear Beckman tubes. The gradients were centrifuged at 26,500 rpm for 90 min in a Beckman SW 28 rotor with No. 119 buckets at 4°C and the virus bands were collected with a 10ml syringe attached to a bent needle. The virus bands were pooled, diluted with PBS and repelleted in 1 x 3 1/2 inch ultraclear tubes at 26,500 rpm at 4°C in a SW 28 rotor with No. 116.8 buckets. The pellets obtained were resuspended by sonication in 1.5ml PBS and carefully layered unto fine sucrose gradients made up with 5.5ml of 45% sucrose and 15% sucrose mixed with a vacuum pump in 9/16 x 3 3/4 inch ultraclear tubes. The gradients were centrifuged at 39,500 rpm for 45 min at 4°C in a SW 40.1 rotor with No. 106.2 buckets. The virus bands were recovered as before into and centrifuged as before in SW

40.1 rotor at 39,500 rpm for 60 min at 4°C. The pellets were resuspended by sonication in 1ml Tris-saline and stored at 4°C for use within 2-3 days or at -20 or -70°C until required. The concentration was determined with Philip Scan spectrophotometer using the method of Bradford (1976).

2.2.7 Analysis of virus polypeptides and cleavability of haemagglutinin glycoprotein in tissue culture

The gene products (polypeptides) of the virus isolates were analysed in 9 different monolayer cell types: chicken embryo fibroblasts (CEF), lamb testes primary cells (LTp6c) with extended life produced at Pirbright Laboratory, Mardin Darby canine and bovine kidney cells (MDCK), (MDBK), African green monkey kidney cells (Vero), baby hamster kidney cells (BHK), Rim suino 2 swine kidney cell (RS2), rhesus monkey kidney cells ($LLCMK_2T$) and BSC40.

2.2.7.1. Preparation of CEF monolayer cells

All procedures were carried out under strict sterile conditions and all solutions were warmed to at about 37°C before use. Embryonated hen eggs were disinfected with methylated spirit before use. Thirty 10-11 day old embryos were removed from the eggs and placed in a petri dish containing sterile PBS. The heads and viscera were carefully removed and discarded. The remaining embryos were washed twice in PBS, placed in a 60ml syringe and macerated by forcefully pushing them through the burr into a 250ml conical flask. The macerated embryos were then washed twice with PBS containing trypsin

to a final concentration of 0.025%. The suspension was stirred at 37°C for 20 min and the resultant emulsion was filtered through a sterile muslin into graduated 50ml centrifuge tubes. The remaining cellular material was restirred in PBS-trypsin at 37°C for 20 min and filtered through muslin into graduated tubes. All the tubes were then centrifuged at 2000 rpm for 10 min at 4°C in a bench top Central 4R or Denley centrifuge. The pellets obtained were resuspended in 30ml Medium 199 growth medium with 10% adult bovine serum (ABS) to a final volume equivalent to 1ml/embryo giving a full concentration of cells as working stock. A 1:30 dilution of this cell suspension was made and 15ml were dispensed into each 15ml tissue culture dishes or 100μl/well into 96-well flat bottom tissue culture plates to give a confluent monolayer of CEF in 24 hr. However, volumes and dilutions used were adjusted according to the size of culture dishes and plates used, the type of analysis and the desired length of incubation of the cells. The cells were then incubated at 37°C in an atmosphere of 5% CO_2.

2.2.7.2. Preparation/splitting of other monolayer cell types

Roller bottles or flasks of cells stored at -70°C were thawed at room temperature and cells were seeded from a 75ml tissue culture flask and split into seven or eight 25ml flasks, incubated as above for about 2-3 days to grow and become confluent. The cells were then split from the 25ml flasks into 96-well plates for analysis of virus polypeptides and cleavability of HA. The growth medium was decanted from each 25ml flask and the cells were rinsed thrice with Ca^{++} and Mg^{++} free PBS and once with 2ml Versen-trypsin, 2ml of which were then dispensed into each flask which was rocked briefly to ensure

contact with the cells and incubated at 37°C + 5% CO_2 for 10 min with the flask covered, during which time the monolayer cells were detached from the flasks. The cells were resuspended in Versen-trypsin in 10ml pipettes by pipetting up and down several times followed by aliquoting the cell suspension in pre-determined percentages into sterile plastic universal bottles marked for each cell type. Each cell suspension was made up to 12ml with appropriate maintenance/growth medium. The cells were thoroughly dispersed by pipetting up and down several times or brief votexing and 100µl of each cell suspension were dispensed into the wells of 96-well plates marked for each cell type followed by incubation as above for 1-2 days or 2-3 days until they were confluent to be used for analyses.

2.2.7.3. Analyses of virus polypeptides and HA cleavability.

The procedure was carried out in sterile 96-well flat-bottom tissue culture plates as described by McCauley and Penn (1990). Tissue culture medium was removed from the cells which were rinsed with warm PBS and growth medium. The cells were then infected with 25µl of infectious AF of each virus isolate and known control influenza A viruses and left at room temperature for about 30 min with rocking every 5-10 min. The cells were then rinsed with warm Earle's salt followed by addition of 50µl per well of warm Earle's salt and incubation at 37°C + 5% CO_2 for 6-8 hr. Cleavability of the HA and viral protein synthesis were investigated by pulse-chase experiments by labelling the different cell types in serum-free medium at 6 hr post infection for 10 min with ^{35}S-L-Methionine (1232.7 Ci/mmol) at 100µCi/µl in Earle's salt and chasing with medium

containing 10mM methionine for 15-30 min. Each pulsed well was then rinsed with cold PBS and the chased wells with 10mM methionine followed by addition of 20μl per well of protein gel sample buffer (8M urea, 2% SDS, 2% 2-mercaptoethanol pH 6.8, 10mM Tris-HCl pH 7.4, 0.05% bromophenol blue). The plates were either electrophoresed immediately or stored at -20°C until required. Electrophoresis of infected cell lysates was carried out on 12.5 or 15 % sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE) as described by Laemmli (1970) under reducing conditions.

2.2.7.4. SDS-polyacrylamide gel electrophoresis of virus polypeptides

SDS-PAGE was carried out using a discontinuous pH gradient and SDS. The resolving gel slab was 1mm tick and was cast in 20cm x 20cm glass gel plates. Stock solutions of 30% acrylamide and 1% bisacrylamide, 10% SDS and 1.5M Tris-HCl pH 8.7 were mixed in a 12.5% or 15% acrylamide with or without urea. A 5% acrylamide and 0.13% bisacrylamide stacking gel of about 2-3cm long was cast on top of the polymerised resolving gel with 5mm, 24 well combs. 1M Tris-HCl pH 6.8 was used for the stacking gel thus giving a pH gradient between the two gel slabs both of which were polymerised with 0.1% ammonium persulphate (APS) and 0.1% N-tetramethylethylenediamine (TEMED). The protein gels were not pre-run and samples in protein gel buffer were heated at 80°C covered in an oven for 10 min before loading. Gels were run for 3-5 hr at about 50 mAmps constant current in 2X running buffer made up of 28.8gm glycine, 6.06gm Tris and 0.1% SDS in a 1 litre of deionised water. Running of the gel was stopped just before the bromophenol blue front reached the bottom of the gel plates. Gels

were stained in 40% methanol, 10% glacial acetic acid, 50% deionised water and 0.5gm Coomasie blue for at least 1 hr and fixed (destained) in 40% methanol and 10% acetic acid in deionised water for at about 30 min. They were then vacuum dried onto 3mm Whatman chromatography paper. Radioactivity on wet and dried gels was monitored with the Radman monitor model 5-101 E.

2.2.7.5. Autoradiography

Dried gels were exposed to fast and sensitive blue X-ray films at room temperature without an intensifying screen. Gels and films were placed in brown or dark envelopes or cardboards before being tucked into film cassettes. Exposure times varied greatly depending on the amount of radioactivity present in the gel but were initially overnight followed by longer exposures of 3 days to 1-2 weeks or more. X-ray films of gels were then viewed on an illuminator.

2.2.8. Production of antisera against the new virus isolates

Polyclonal antisera were raised against each of the new virus isolates in three outbred rabbits by standard methods. Each rabbit was given 200μg in 1ml of purified virus of each isolate emulsified in 1ml of complete Freund's adjuvant (kindly provided by Dr. A.I. Daneji) by injecting 1ml into the foot pads and the other 1ml into the thigh muscles. The rabbits were boosted by intramuscular injection of 200μg of purified virus emulsified in Freund's incomplete adjuvant 2 weeks later and bled for serum 2 weeks after the booster injection. Harvested sera were stored at -20°C until used for antigenic cross-reactivity analysis in HI assays.

2.3. RESULTS

2.3.1. Virus isolation in eggs and MDCK cells

Antigenic cross-reactivity analyses showed that the samples positive for virus by HA were influenza A viruses. Three influenza A viruses were independently isolated in eggs and MDCK cells from a total of 1,076 samples obtained from symptomatic and asymptomatic equines from various parts of the country giving 0.28% positive samples. All the three viruses were isolated from samples collected from horses at Ibadan in 1991 during the annual polo tournaments although horses came from many parts of the country and were thus named regardless of which part of the country the horses came from. No virus was isolated from the donkeys. Initially, haemagglutination of 1% rbc was observed in 1 or 2 wells in the positive samples. Subsequent passages gave higher HA titers until the forth or fifth passage (second and third passage from the working stocks) which gave a multiplicity of infection (m.o.i.) of 10 in the egg isolates indicating a degree of adaptation of the equine viruses to chicken chorio-allantoic membranes while m.o.i. of >10 was observed by the third passage with viruses isolated in MDCK cells. All samples negative by HA were discarded after 6 passages.

None of the samples collected from the donkeys was positive for virus.

Cytopathic effect was observed from the third day of incubation of MDCK cells in 8 flasks (Plate 1) and was characterised by cell membrane blebbing and condensation of cellular materials resulting in cell shrinkage in 3 flasks (Plate 2). The nuclei of the

Plate 1. Cytopathic effect in virus infected MDCK monolayer cells on days 3-4 post infection. Note the complete destruction of the monoplayer cells (white areas) interspersed with cells that have lost their architecture.

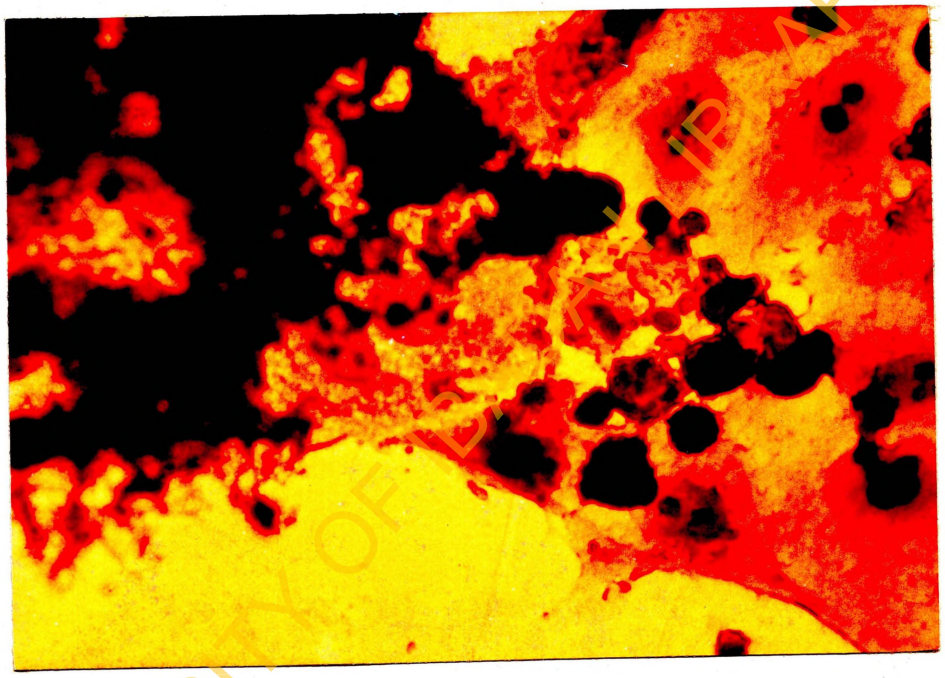
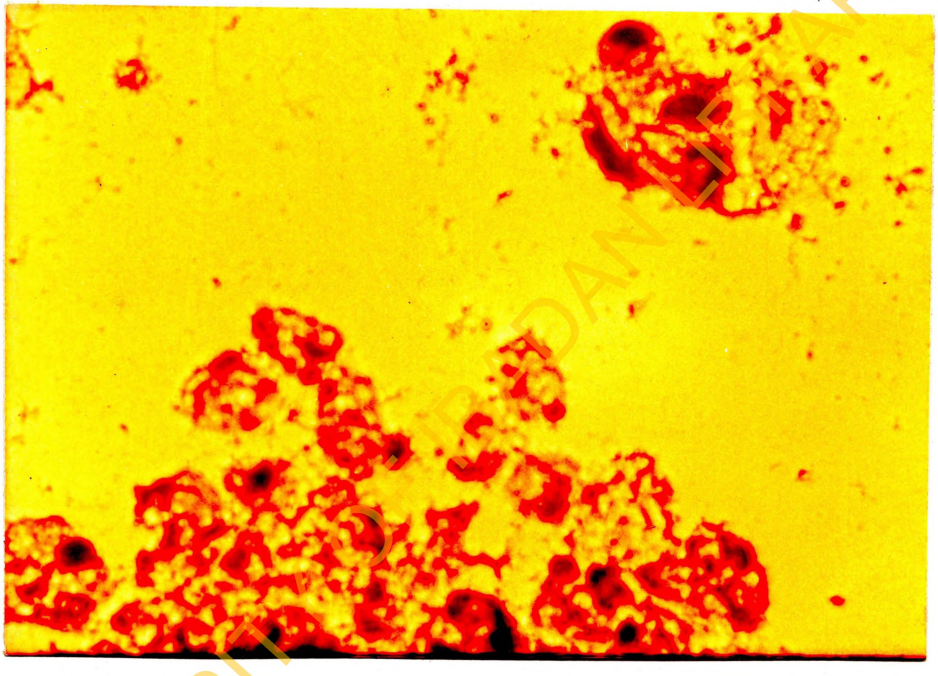


Plate 2. Cytopathic effect in virus infected MDCK monolayer cells on days 5-7 post infection. Note the absence of cells (blue area) and cell debrils left from the dead cells.



cells were also fragmented . Only supernatants from these three flasks agglutinated chicken and guinea-pig rbc and corresponded to the three isolates from eggs. Samples from the other 5 flasks that showed CPE failed to agglutinate rbc after 3 passages in HeLa cells probably implying that they were not influenza viruses. There was neither clustering nor syncytial formation in the HeLa cells, neither were there any intranuclear inclusion bodies observed in the stained cells. The implication of these observations is that no other respiratory disease viruses were present in the clinical samples that were positive for influenza viruses although other methods and tests were not carried out to really confirm this since this was beyond the scope of the present studies. The CPE observed in these other 5 flasks was probably due to contamination.

The three equine influenza virus isolates were respectively named A/Equine/Ibadan/4/91, A/Equine/Ibadan/6/91 and A/Equine/Ibadan/9/91 according to the recommendations of WHO and the International Committee on Virus Taxonomy.

2.3.2. Purification and concentration of purified viruses.

The three virus isolates were successfully purified by differential sucrose gradient centrifugation. Their protein concentrations were respectively 24mg/ml, 20.6mg/ml and 21.2mg/ml for A/Eq/Ib/4/91, A/Eq/Ib/6/91 and A/Eq/Ib/9/91.

2.3.3 Virus typing and antigenic analysis

2.3.3.1 Virus typing

The three isolates did not react with the antisera against the other respiratory disease viruses but showed positive reactions to antisera against the NP of A/Peuto Rico/8/34, A/Eq/Miami/63 and A/Eq/Prague/56. They also failed to react with antisera to the NP of B/Lee/40 and C/Ann Arbor/50. This implied that the isolates were influenza virus type A.

2.3.3.2. Haemagglutination inhibition tests (HI)

Table 3 shows the HI titers of the three virus isolates with Mabs while the HI titers with polyclonal antisera are shown in Table 2. HI results indicated that the 3 new isolates were H3 HA subtype. Mabs to A/Eq/Miami/63 obtained from Mill Hill, London showed greater cross-reactivity with the Ibadan isolates than A/Eq/Miami/63 Mabs obtained from Memphis, Tennessee while Eq/Tennessee and Eq/Kentucky Mabs showed greater cross-reactivity with Eq/Ibadan/4/91 and Eq/Ibadan/6/91 viruses than with Eq/Ibadan/9/91 virus. On the other hand, Eq/Fountaineblue/79 and Eq/Iowa Mabs showed greater cross-reactivity with Eq/Ibadan/6/91 and Eq/Ibadan/9/91 viruses than with Eq/Ibadan/4/91 virus which gave >10 fold lower titers than the former two viruses. Similar reactivity patterns were observed with polyclonal antisera against homologous and heterologous viruses. Antisera to Eq/Miami/63, Eq/Fontaineblue/79, Eq/Switzerland/76 and Eq/Newmarket/76 cross-reacted to much lower titers with Eq/Ibadan/4/91 virus than with the other two isolates while Eq/Ibadan/9/91 showed very high cross-reactions to these antisera. The

reactivity titers of the 3 isolates with monospecific antisera to equine-2 HA were also very high indicating that they were H3 HA subtype viruses.

Comparison among the 3 isolates in their reactivity with both Mabs and polyclonal antisera showed that although they were all H3 HA subtype, they appeared to be antigenically distinct from each other. A/Eq/Ibadan/4/91 and A/Eq/Ibadan/6/91 seemed to be more closely related to each other than to A/Eq/Ibadan/9/91 virus, implying that some degree of antigenic variation occured among the 3 viruses as shown in Figs. 11 and 12.

Table 2. Haemagglutination inhibition (HI) titers of Ibadan equine influenza virus isolates with polyclonal antisera.

Virus	Miami/ 63 (H3N8)	Ft- bleu/ (H3N8)	Switz/79 (H3N8)	Nmarket /76 (H3N8)	Ib/4/91 (H3N8)	Ib/6/91 (H3N8)	Ib/9/91 (H3N8)	Praque /56 (H7N7)
A/Eq/Ibadan 4/91	160	80	80	160	1280	1280	640	20
A/Eq/Ibadan/6/91	640	160	320	320	1280	2560	160	< 20
A/Eq/Ibadan/9/91	640	320	320	640	640	160	1280	< 20

Rabbit antisera against A/Eq/Miami/63, A/Eq/Ftbleu/79, A/Eq/Swtizerland/79, A/Eq/Newmarket/76 and A/Eq/Prague/56 were from Allan Douglas, Nat. Insti. for Medical Research, Mill Hill, London NW7 1AA, U.K.

Table 3. Haemagglutination inhibition (HI) titers of Ibadan equine infuenza virus isolates with monoclonal antibodies.

Monoclonal Antibody / Virus	Miami/1/63[1]				Fbleu/1/79		Tennessee/5/86[2]				Kentucky/81[2]				Iowa/86[2]	
	H1	H3	H4	H6	H1	H2	2/1	4/1	14/1	22/1	1/1	2/1	3/1	4/1	1/1	2/1
IBADAN/4/91	5120	5120	2560	2560	640	640	5120	320	2560	640	1280	320	40	80	40	80
IBADAN/6/91	5120	2560	5120	5120	2560	2560	5120	1280	2560	2560	2560	2560	320	320	1280	320
IBADAN/9/91	2560	1280	640	5120	5120	1280	1280	160	320	320	320	320	80	80	5120	2560

1. From Allan Douglas, National Institute for Medical Research, Mill Hill, London NW7 1AA, UK.

2. From Dr. R.G. Webster, St. Jude Children Research Hospital, Memphis, Tennesse, USA.

Fig. 11 Comparison of Influenza Isolates from Ibadan, Nigeria
Use of Monoclonal antibodies

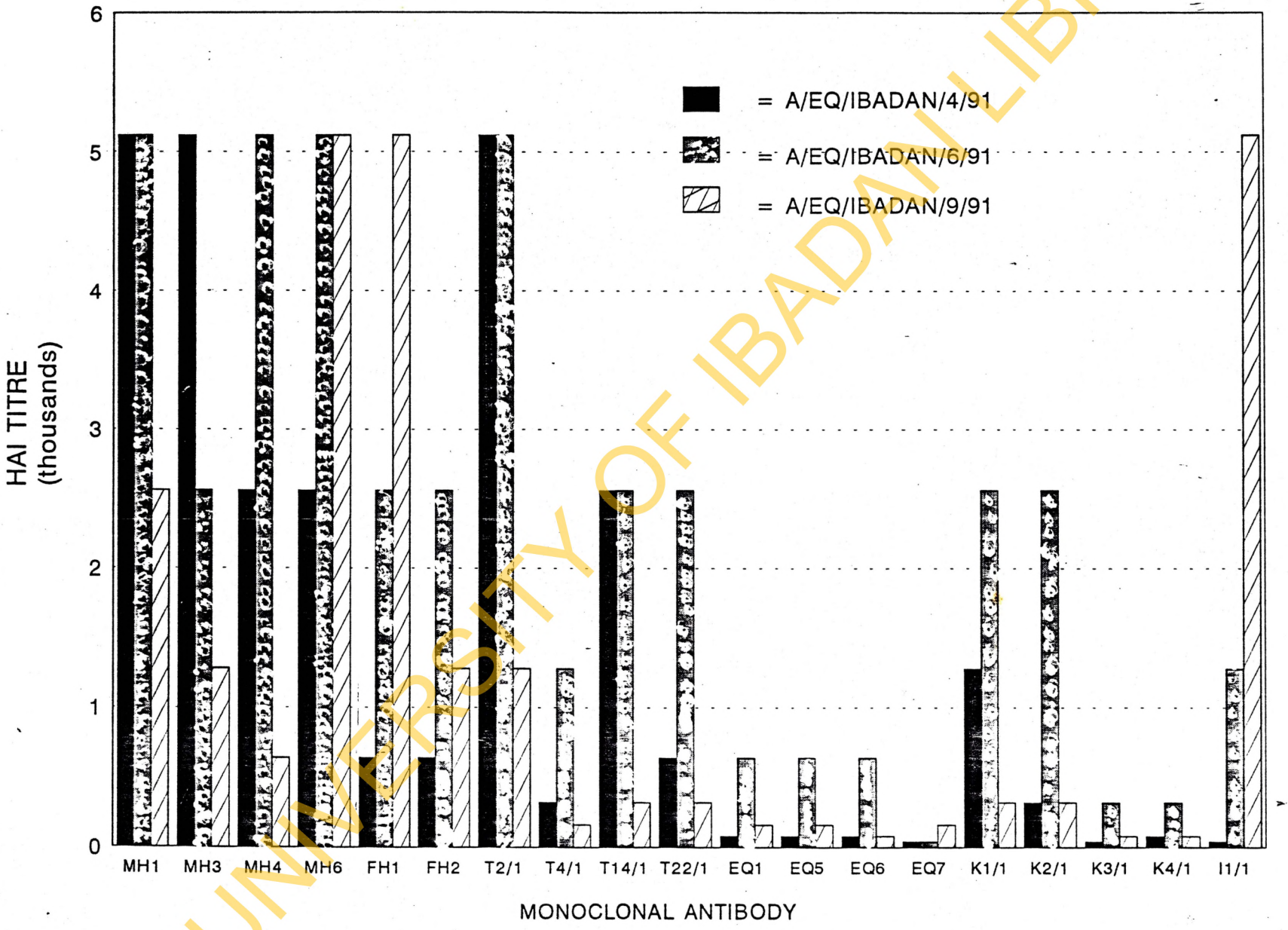
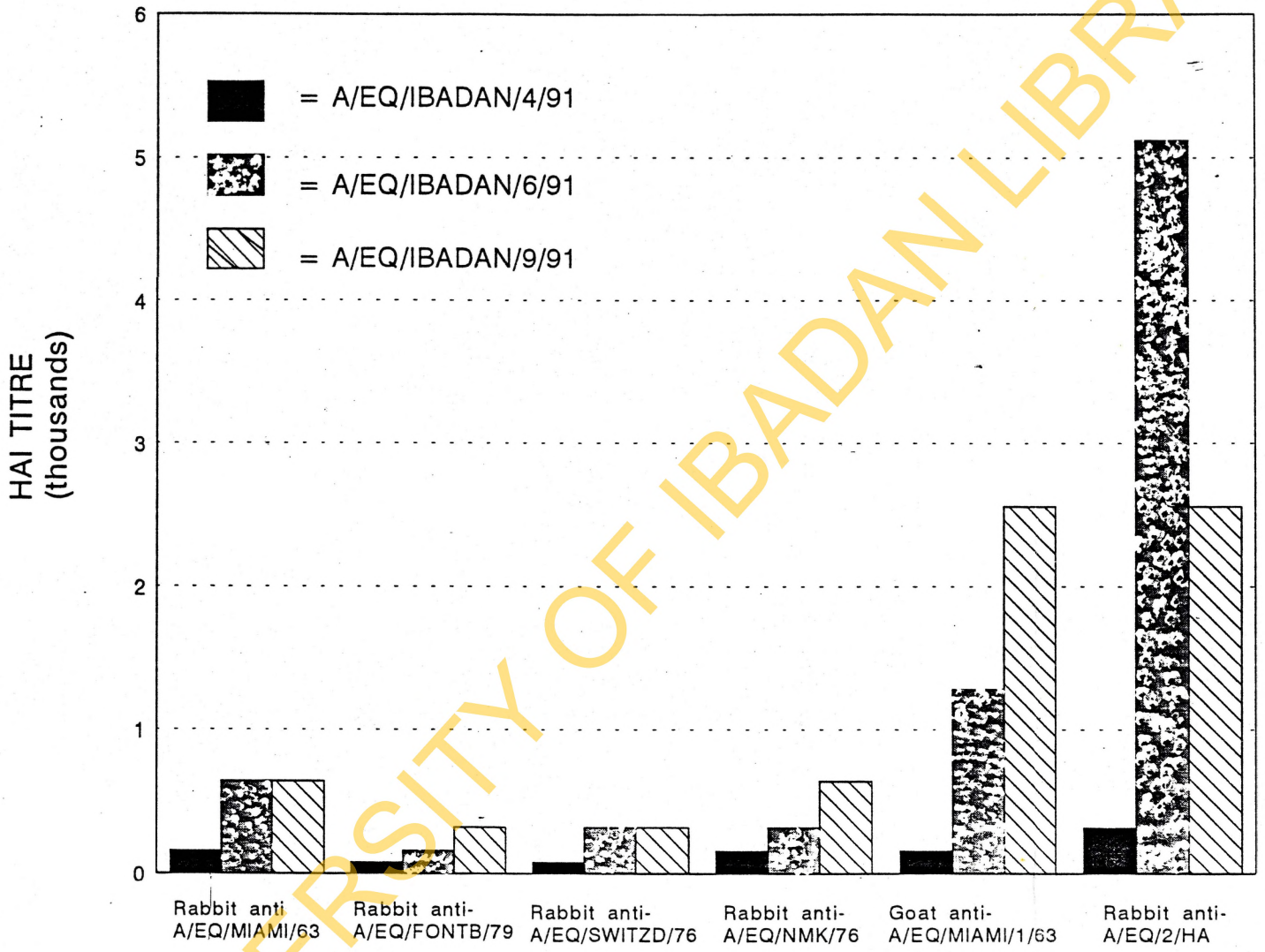


Fig. 12 Comparison of Equine Influenza Isolates from Ibadan, Nigeria
Use of Polyclonal antisera



2.3.3.3. Neuraminidase inhibition assays

The virus dilutions used for the assays were 1:32 for A/Ae/Ibadan/4/91, A/Eq/Newmarket/76 (H3N8) and A/Duck/Memphis/546/74 (H11N9), 1:64 for A/Eq/Ibadan/6/91 and 1:16 for A/Eq/Ibadan/9/91. The results of the NA spot test showed that the 3 new virus isolates were N8 NA subtype which was confirmed by measuring the anti-NA titers of the two reference antisera against an N8 and an N9 viruses in the full NAI assays. The NAI titers are shown in Table 4. Antigenic comparison of the NA subtype of the 3 new isolates with monospecific rabbit antiserum to an N8 equine virus confirmed the NA of the 3 isolates to be N8 subtype.

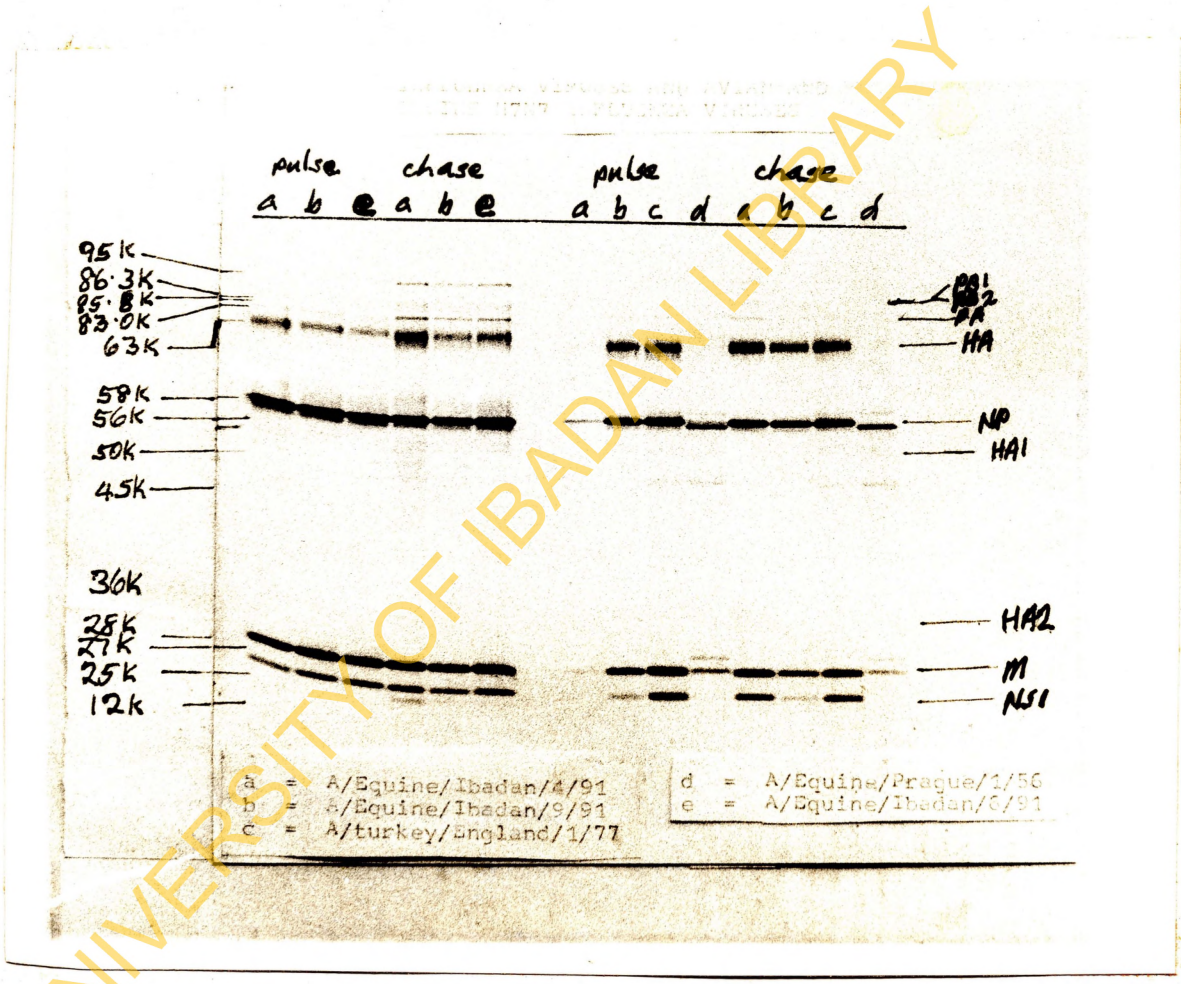
2.3.4. Analysis of virus polypeptides and cleavability of the haemagglutinin glycoproteins in tissue culture

The electrophoretic mobility and pattern of virus polypeptides are shown in Plate 3. The HA glycoproteins of the 3 isolates were not cleaved in any of the cell types used in contrast to that of equine-1 virus, A/Eq/Prague/56 (H7N7). However, some differences were observed in the mobility and pattern of the internal proteins of the 3 virus isolates indicating some degree of heterogeneity. The polymerase proteins and the NP of the 3 isolates differ slightly from the other known control viruses particularly the NP of A/Eq/Ibadan/4/91 which appeared to be avian-like and the NP of A/Eq/Ibadan/9/91 which appeared to be a doubler. These diferences could prove to be of some genetic and epidemiological importance. Studies on detailed molecular characterisation of the isolates in a later chapter would elucidate this.

Table 4. Neuraminidase inhibition (NAI) titers of Ibadan equine influenza virus isolates.

Virus	NI titres to rabbit antiserum to A/Eq/Newmarket/7/6 (H3N8)	NI titres to rabbit antiserum to A/Duck/Memphis/546/74 (HIIN9)
A/Eq/Ibadan/4/91	12800	< 100
A/Eq/Ibadan/6/91	10000	< 100
A/Eq/Ibadan/9/91	6400	< 100
A/Duck/Ireland/83 (H5N8)	10000	< 100

Plate 3. Influenza virus encoded polypeptides.
Pulse-chase labelling of chicken embryo fibroblast monolayer cells infected with influenza A viruses using 35^S methionine to show the synthesised virus encoded polyeptides. Note that the HA of A/Eq/Prague/56 (H7N7) virus is cleaved into HA1 and HA2 polyeptides in tissue culture in contrast to those of Ibadan viruses a, b and e (H3N8) and the non-pathogenic avian virus A/Turkey/England/1/77 (H7N7).



2.3.5. Production of polyclonal antisera angainst the virus isolates.

The results of antigenic cross-reactivity of the virus isolates against homologous antisera are shown in Table 2 and showed that antisera production was very good.

2.4 DISCUSSION

Influenza viruses of man, other mammals and birds pose a serious threat of disease throughout the world and epidemiological/epizootiological surveillance is an important activity aimed at limiting the spread of infection and disease. Two subtypes of influenza A virus cause disease in equines: equine-2 virus which is prevalent in many countries in the world and equine-1 virus which has not been isolated anywhere for about 15 years (Guo *et al.*, 1992). Equine influenza outbreak has never been reported in Nigeria or in tropical Africa to my knowledge. There is therefore little or no information on the occurence, isolation and characteristics of the virus as well as the clinical manifestation and epidemiology of the disease despite the regular movement of horses into this region from other parts of the world. This work describes the first equine-2 (H3N8) viruses isolated and characterised in tropical Africa although equine-2 viruses had previously been isolated from the African continent: the first was in Algeria in 1972 (A/Eq/Algeria/72) in temperate North Africa and the second was in Johannesburg in 1986 (A/Eq/Johannesburg/86) in temperate South Africa in the Southern Hemisphere both of which were outside the tropics. The above results indicate the occurence of equine influenza viruses in Nigeria and establish an epidemiological record for this environment on which further work could be built. Although the number of samples from which viruses

were isolated was very small (0.28%) compared with the total number of samples collected from equines from various parts of the country, the isolation of these viruses from animals resident in Nigeria is of epidemiological significance. These animals comprised indigenous horses and donkeys as well as exotic breeds some of which had recently been imported into the country from neighbouring countries and from Europe and South America where there have been recent outbreaks of equine-2 influenza infections (Wood and Mumford, 1992; Lai *et al.*, 1994). Most of the donkeys are used for transportation of wares and personal effects although no virus was isolated from any of them while most of the horses are used for polo and local racing. The isolation of these viruses from Nigeria emphasises the prevalence of equine-2 influenza virus in many parts of the world and confirms the increase in the incidence of this virus as evidenced from reports cited earlier. Some of the horses sampled showed clinical signs of respiratory disease suggestive of influenza. It is probable that these animals had a primary exposure to the virus. It is however not known if these viruses were already circulating in the indigenous equine populations or if they were brought in by recently imported animals, although antibodied to equine-1 and 2 viruses had been demonstrated in the sera of some horses collected in the late 1970s (Dorojaiye and Denya, personal communication). Since there is no evidence that influenza viruses persist for extended period in any individual (Webster and Kawaoka, 1994), it is unlikely that any viruses that circulated in equines in the late 1970s in Nigeria could still be circulating among the animals sampled in the present studies. The most likely source of the present isolates would be recently imported animals. There is no established routine vaccination programme for horses in Nigeria against virus infections

although some previledged horse owners ocassionally import already vaccinated animals or import vaccines against some viral diseases of horses and seek veterinary assistance in administering the vaccines. It is probable that the antibodies detected by Durojaiye and Denya were from vaccinated animals. It is equally probable that the present virus isolates could have originated from vaccine strains. However, antibodies to equine-2 influenza viruses were detected in equine sera collected between January 1989 and December 1993 which indicated about 15% prevalence rate (Adeyefa *et al.,* 1995). The inability to isolate viruses subsequent to 1991 could be due to the prevailing high antibody titer in some of these animals following previous or recent exposure to equine influenza viruses or the virus antigens. It is also not unusual for occasional disappearance of circulating viruses from immuned host species. Viruses were not isolated in the U. K. for about 10 years until the 1989 outbreaks of disease (Binns *et al.*, 1993). In addition, the ability to isolate viruses from samples stored from 1989 - 1995 could be due to irregular electricity supply in Ibadan which might have adversely affected the survival of viruses in the samples and thus limited the changes of isolating viruses from these samples. It is hoped that nucleotide sequence analysis in a later chapter could indicate the origin of the virus genes and the likely source of the viruses.

The HAs of the three virus isolates were not cleaved in tissue culture in contrast to equine H7 HAs (Gibson *et al.*, 1992). *In-vivo* cleavage of the HA polypeptide into HA1 and HA2 is required for virus infectivity (Klenk *et al.*, 1979). It is related to the virulence of avian influenza A viruses and two structural features are involved: a series of basic amino acids at the cleavage site and an oligosaccharide side chain nearby (Kawaoka,

1991). Similarly, Gibson *et al.*, (1992) have demonstrated a tetrabasic amion acid cleavage site in equine H7 HAs in tissue culture in addition to a nine amino acid insertion prior to the tetrabasic sequence. Introduction of a series of basic amino acids at the cleavage site of human H3 HAs in tissue culture renders the HAs susceptible to cleavage enzymes (Kawaoka, 1991) but this has not been reported for equine H3 HAs. Lack of this series of basic amino acids and the nine amino acid insertion at the cleavage site of equine and human H3 HAs as well as those of avirulent avian virus HAs could be responsible for the non-cleavability of the present isolates' HAs in any of the cell type used in this study. Non-cleavability of equine H3 HAs or avirulent avian HAs in any of the nine cell types used in this study implies that the virus-host system used has no effect on HA cleavability in tissue culture *per se* but rather HA cleavability in tissue culture is an intrinsic property of the HAs as shown by equine H7 and pathogenic avian H5 and H7 HAs in tissue culture. However, *in-vivo* cleavage of equine H3 HAs occurs in natural infections probably due to the abundance and ubiquity of cellular proteases capable of cleaving the HAs of virulent viruses.

Examination of the other virus polypeptides showed some heterogeneity among the three isolates particularly in the polymerase (P) and the NP proteins which could be of some genetic and epidemiological importance but elucidation of this would have to wait for nucleotide sequence analysis in a later chapter.

Antigenic analysis of the virus isolates with Mabs and polyclonal antisera showed that the three viruses appeared to be antigenically divergent. Their reactivity to Mab panels and the various antisera used suggests that the viruses were antigenically distinct.

Although they were all equine H3N8 subtype, they showed low to moderate antigenic cross-reactivity with polyclonal antisera and low to high cross-reactivity with Mabs. This antigenic divergence is further reinforced by the heterogeneity in their internal protiens. These observations indicate that antigenic variation also occurs among equine H3 HA molecules and that equine H3 viruses with antigenically different HA molecules could co-circulate. Kawaoka *et al.*, (1989a) made similar observations in their study of equine H3N8 viruses isolated between 1963 and 1987. The implication of these observations is that like human H3 HA molecules, equine H3 HAs have evolved by progressive antigenic variation and divergence probably as a result of selective pressure or some mutational changes in their HA genes which nucleotide sequence analysis and amino acid deduction may confirm. The above results demonstrate intra and inter-strain differences which have potential significance in the production of high yield influenza virus reassortants that could be used in vaccines against antigenic variants emerging in nature. The differences observed in the isolates' reactivity in HI tests could be ascribed to pleitropic differences in the viruses themselves possibly due to the greater antigenic diversity of egg-grown viruses (Robertson *et al.*, 1991) which were used for the analysis. Intra-isolate antigenic heterogeneity has also been observed in human influenza A virus isolates (Katz *et al.*, 1990).

In this study, parallel HI tests were not carried out with the well characterised laboratory strains against which the Mabs and polyclonal antisera used were raised to preclude cross-contamination because the subsequent strategy used for molecular characterisation of the viruses involved extensive use of PCR which is highly susceptible

to cross contamination. Previously, equine H3 viruses have been shown to be antigenically divergent (Kawaoka *et al.*, 1989a) but viruses from 1983 to 1987 exhibited an antigenic phenotype that regained reactivity to Mabs directed against viruses isolated in 1963, 1979, 1981 and 1986. Although in the present study the Mab panels used were not identical to those used by Kawaoka *et al.*, (1989a), the broad reactivity of these Ibadan isolates suggests that they were similar to the viruses isolated in USA between 1963 and 1987. The variation in antigenic reactivity to the Mabs and antisera appears to be at the limit of significance (an eight fold difference in titer with only one Mab). Of all the 20 Mabs used, none failed to show any reactivity to any of the three isolates. This result is not unexpected since, although antigenic variation has been recorded as far back as the first isolation of an equine-2 subtype virus in 1963, viruses isolated in USA in the mid-to-late 1980s demonstrated reactivity to Mabs raised against the prototype virus (A/Eq/Miami/63) whilst other strains isolated in the seventies, 1981 and 1982 showed no such reactivity. The pattern of reactivity in the results above could be taken as an indication of a close relationship between the USA equine-2 viruses and the tropical African Ibadan isolates described here. However, this conclusion would not differentiate between an American or South African origin for these Ibadan isolates, since the HA gene of the virus isolated from Johannesburg in South Africa in 1986 differed from the HA gene of the virus isolated in Kentucky in 1986 by two base changes and one amino acid change. The Kentucky virus was the origin of the Johannesburg virus (Kawaoka and Webster, 1989). The likely origin of the genes of these Ibadan isolates would be indicated by genetic analysis in a later chapter.

CHAPTER 3

VIRUS INFECTIVITY, ANTIGENICITY AND IMMUNOGENICITY

3.1 INTRODUCTION

Viruses are unable to replicate on their own but must enter a living host cell and use the host cell macromolecular machinery and energy supplies to replicate at the expense of the host cell (Knipe, 1990). The injurious effects of viral replication on the host cell are one of the basic causes of viral disease to which the host responds immunologically. In influenza infection, it requires only one virus particle to infect a cell and form plaque, thus displaying a one hit kinetic. Virus infectivity is thus determined *in-vitro* by plaque assays in tissue culture and titration of the virus in fertile hen eggs. Infection or immunisation with influenza viruses or vaccines results in production of antibodies directed against various components of the virus. Both humoral and cellular immune responses are induced and they play a major role in the overall immunological response of the host. Virus infection of a cell can lead to any of several possible outcomes such as (a) a non-productive infection in which viral replication is blocked (b) a productive infection which is deleterious to the host, (c) persistent infection in which the cell may survive and continue to produce virus at a low level. In all these there are interactions between virus-encoded macromolecules and host cell components which invariably lead to immunological responses by the host through the activation of B and T lymphocytes to produce antibodies (humoral response) or induction of various T-cell subsets (cellular response).

Lymphocyte mitogens have been widely used to probe events involved in activation, proliferation and differentiation of B and T lymphocytes in rodents and man (Anders *et al.*, 1984) and some viruses have been shown to be mitogenic including vesicular stomatitis virus (Goodman-Snitkoff and McSharry, 1980), Sendai virus (Kizaka *et al.*, 1983) and some human influenza virus strains (Armstrong *et al.*, 1981; Hurwitz *et al.*, 1985). These workers have so far used normal or primed spleen cells from mice and humans in *in-vitro* assays. There is paucity of information on the mitogenic activities of equine influenza viruses using equine peripheral blood lymphocytes (PBL). It is concievable that equine influenza virus glycoproteins could induce mitogenic lymphocyte proliferation *in-vivo* as a part of an overall immunological response of the horse to influenza virus infection since the virus glycoproteins are targets of anti-viral immune response of the host and since mitogenic lympho-proliferation modulates the animal's immunological responses.

Cellular immune response comprises a complex interaction between different cell types and molecules and T-cell induction requires antigen processing, presentation and recognition by T-cell receptors (Berzotsky *et al.*, 1988; Lanzavecchia, 1990). T-cell activation in turn results in the release of lymphokines which induce proliferation and antibody production by B-cells. The present studies are aimed at determining the infectivity, *in-vitro* and *in-vivo* mitogenicity, antigenicity and immunogenicity of the three Ibadan equine influenza virus isolates as well as the protective antibody response that could possibly result from such antigenic and immunogenic lymphocyte proliferation iusing

infectious viruses, intact and denatured virus glycoproteins as immunogens for a better understanding of the primary response of equines to a productive influenza virus infection.

3.2 MATERIALS AND METHODS

3.2.1 Virus infectivity

Infectivity of the virus isolates was determined by plaque assays in tissue culture using CEF monolayer cells and by titation of the viruses in embryonated hen eggs.

3.2.1.1 Plaque assay

CEF monolayer cells in 6-well tissue culture plates were infected as described above in chapter 2 with 150μl of 2-fold serial dilutions of each virus using the infectious allantoic fluid (AF) diluted in PBS from 10^{-1} to 10^{-9} dilution. The plates were then incubated at room temperature of 22-25°C for 1 hr being rocked every 10 min. The inoculum was removed and the cells were overlaid with 3ml per well of maintenance medium containing 8.8% bicarbonate, 4% ABS, 1.5% agar (at 45°C) with or without 0.04% trypsin. On setting the plates were either incubated at 37°C + 5% CO_2 for 3-5 days or for 1 hr before being sealed in plastic bowls and submerged in a 41.5°C water bath to determine if some temperature sensitive mutants would form plaques water bath for 3-5days. The plates were stained after incubation with 0.1% neutral red dye and further incubated at 37°C for 4 hr while the stain penetrated the cells. The plates were checked for plaques which were then counted. The results were expressed in plaque forming unit (PFU).

3.2.1.2. Virus titration in embryonated hen eggs

Serial 2-fold dilutions of each virus isolate AF were made in PBS containing antibiotics and supplemented with 0.2% ABS and 100μl of each dilution for each virus were inoculated into the allantoic cavities of three 10-11 day old fertile hen eggs which were then incubated at 37°C for 48 hr. The AF were harvested and tested for HA of 1% chicken rbc. Virus titers were expresed in 50% egg-infecting dose (EID_{50}) per ml.

The ratio of PFU and EID_{50} was determined for each isolate.

3.2.2. Virus mitogenicity and antigenicity.

3.2.2.1 *In-vitro* mitogenicity: *In-vitro* response of equine peripheral blood lymphocytes (PBL) to the glycoproteins of equine influenza viruses

(a) Viruses and glycoprotein preparation

The viruses used in this study were A/Eq/Ibadan/4/91, A/Eq/Ibadan/6/91, A/eq/Ibadan/9/91, A/Eq/Miami/63, all H3N8 subtype and A/Eq/Prague/56, H7N7 subtype designated as Ib4, Ib6, Ib9, M63 and P56 respectively. The viruses were grown in fertile hen eggs and purified by differential sedimentation through 15-60% sucrose gradients as described in chapter 2. Previoulsly described method of Brand and Skehel (1972) was used for isolating the virus glycoproteins. Breifly, the purified viruses were treated with bromelain (Sigma) and solubilised glycoproteins were isolated by centrifugation through 5-30% sucrose gradient or glycerol gradient as described above. The glycoprotein bands were pooled and concentrated by vacuum dialysis and their protein concentrations were

determined using the method of Bradford (1976) with a Philip scan UV spectrophotometer. The preparations were stored at -20°C until required.

(b) Peripheral bood lymphocytes (PBL)

PBL were obtained as previously described by Adeyefa (1989) from a clinically normal one month old donkey foal whose serum had been tested twice at birth and a month later for influenza virus antibodies by HI and ELISA and found to be negative on both ocassions. Briefly, 40ml heparinised blood were collected aseptically from the jugular vein of the foal and PBL were obtained by density gradient centrifugation on lymphocyte separating medium (Folw Laboratories, Scotland). The cells were washed thrice in minimum essential medium (MEM) containing spinen salts and resuspended at a concentration of 1 x 10^6 cells/ml in RPMI 1640 medium containing 10% fetal calf serum. Cultures in 200μl volumes cotaining 2 x 10^6 cells/ml pipeted into the wells of 96-well flat bottom tissue culture plates. Viability test of the cells was done by trypan blue exclusion method and percentage viability ranged from 89.8 to 99.5%.

(c) Lymphocyte proliferation assays

The glycoproteins were thawed on ice and diluted to concentrations ranging from 0.1 to 200μg/ml. Mitogenic lectins such as concanavilin A (Con A), phytohaemagglutinin (PHA) and poke weed mitogen (PWM) at concentrations of 0.5μg, 1μg and 5μg respectively were used as positive control mitogens to ensure the viability of the cells. The non-radioactive cell proliferation assay was used based on the method of

Mosmann (1983) with slight modifications. Twenty microliters (μl) of varying concentrations of the test glycoproteins and the control mitogens were pipeted into the wells of the culture plates and 200μl of PBL suspension were added to marked wells. Cultures containing only PBS in triplicates as the test mitogens served as unstimulated control cultures. The plates were then incubated at 37°C in an atmosphere of 5% CO_2 for 48-72 hrs. Lymphocyte proliferation was measured by adding 15μl of 50mg/ml MTT solution [3-(4,5-dimethylthiazol-2-yl)2,5,-diphenyl tetra-zoliumbromide] to culture wells and incubating the plates for 4hr as before. This was followed by adding 100μl/well of dimethyl formamide (Sigma), shaking the plates on an orbital shaker and further incubating them for 1 hr as before. The plates were then shaken to mix the contents and the absorbance at 570nm was measured in a Dynatech 5000 ELISA plate reader with a 630nm reference filter. The results were expressed as stimulation indices (SI):

$$\text{S.I.} = \frac{\text{absorbance of stimulated cultures}}{\text{absorbance of unstimulated control cultures.}}$$

Parallel lymphocyte counts were also determined in the lymphocyte counting chamber of Haemalog D particle counter (Technicon Instrument, Hants, U.K.) to monitor lymphocyte proliferation determined by the non-radioactive cell proliferation assay. The results were expressed as SI which represents the lymphocyte counts of stimulated cultures divided by the counts of unstimulated cultures.

3.2.2.2. In-vivo mitogenicity and antigenicity.

(i) Injection of animals with virus glycoproteins and lymphocyte proliferation assays.

Three animals comprising a yearling colt, a 6 month old colt foal and a 4 month old donkey foal were used as test animals while two adult female donkeys served as control animals. Sera from each animal had been previously tested for influenza virus antibodies on two ocassions and found to be negative on both ocassions. Two aliquots of 40ml heparinised venous blood were collected from each animal. From one aliquot, PBL were obtained as before while the other aliquot was stored at 4°C. Each of the three test animals was then injected intravenously (iv) with 5ml of the respective glycoproteins of the three new isolates at a concentration of 10μg/ml. The PBL from the first aliquots of blood were then assayed as unstimulated cultures with PBS as before. Seventy two hr post iv glycoprotein injection, 40ml of heparinised venous blood were collected from each animal and the PBL from the test animals were assayed as stimulated cultures while the PBL from the two control animals and from the aliquots stored at 4°C were assayed as unstimulated control cultures. Subsequently, 40ml of blood were collected from each animal at 1, 2, 4 and 6 weeks and PBL obtained from the test animals were assayed as stimulated cultures while PBL from the control donkeys were assayed as unstimulated control cultures. The plates were read in the Dynatech ELISA plate reader and the results were expressed as SI as above.

(ii) Serology

Blood was also collected for sera at the same time the blood for lymphocyte proliferation assays was collected and also at 12 weeks post iv glycoprotein injection. Haemagglutination titrations of the viruses and HI tests were performed in microtiter plates as described earlier in chapter 2 using both AF virus and virus glycoproteins. Rabbit anti sera to A/Eq/Fontaineblue/79 (F79), H3N8 subtype and A/Eq/Prague/56, H7N7 subtype were used as positive control sera while serum from a pathogen-free guinea-pig served as negative control serum. All the sera used were treated with potassium-M-periodate. Serial two-fold dilutions of test and control sera were reacted in neutralisation tests in 96-well tissue culture plates with AF virus antigens containing approximately 10^1EID_{50} using Vero monolayer cells incubated at 37°C for 48 hr and checked for CPE or neutralisation of the viruses.

Counter-current immunoelectrophoresis (CIEP) and double immunodiffusion tests were also performed by standard methods on the sera to check for precipitating antibodies.

HI titers were expressed as the reciprocals of the highest serum dilution inhibiting 4 HA units of the virus while the neutralisation (NT) titers were expressed as the reciprocals of the highest serum dilution that neutralised 10 EID_{50} of the viruses. For HI and NT tests A/Eq/Fontaineblue/79 was used as positive control antigen.

(iii) Experimental infection of animals

All the animals were clinically examined twice a week for a further 3 months for signs of influenza and other infectious diseases. At 6 months post iv glycoprotein injection

each of the three test animals was challenged with an intranasal inoculation of AF virus containing about 10^9 EID_{50} of the respective Ibadan isolate as previously exposed (or immunised) animals. One of the control donkeys was also challenged with Ib4 virus as previously unexposed (or unimmunised) animal while the other control donkey served as uninfected control. The challenged animals were housed in the uncompleted Veterinary Teaching Hospital in-patient building to separate them from other animals. They were examined for 3 weeks for signs of influenza and naso-pharyngeal swabs were also obtained for virus recovery. Venous blood was collected for sera at 5 and 14 days post challenge. A 1:10 dilution of the swabs from each animal was inoculated into BHK monolayer cells in 25ml tissue culture flasks incubated at 37°C for 4-7 days for virus recovery. HA tests were performed on the culture supernatants of the cells which were passaged thrice.

3.2.3 Virus immunogenicity

(A) Characterisation of proliferating lymphocytes

PBL were obtained and 200μl cultures were stimulated with 100μg of Ib4 and P56 virus glycoproteins as above. Ten milliliters of cell suspension were passed over nylon wool thrice to deplete them of B-cells to the bearest minimum (Anders *et al.*, (1984) found that passage over nylon wool reduced B-cell population to 1.4%). Two hundred μl of T-cell enriched suspension at 2 x 10^6 cell/ml were then stimulated as before. Two sets of triplicate wells of cells with only PBS and culture supernatant from mock infected BHK cells served as unstimulated control cultures to ensure that cell stimulation was induced by the virus glycoproteins. Lymphocyte proliferation assays were then performed as above

and expressed as SI.

(B) Induction of virus glycoprotein mitogenicity and immunogenicity

PBL were obtained from an influenza naive donkey for *in-vitro* simulation with AF virus antigen and 100μg each of intact and denatured virus glycoproteins. Denaturation was achieved by heating the glycoproteins at 90°C for 2min for in-vivo lymphocyte stimulation and by adding 1% SDS for *in-vitro* stimulation. Five milimiters of 10μg/ml of intact and denatured glycoproteins were injected iv into each of 2 influenza naive donkeys from which PBL were obtained post injection. Lymphocyte proliferation assays were then carried out on both *in-vitro* and *in-vivo* cultures. In addition to the negative control cultures with PBS, culture supernatant from mock-infected BHK cells and 5μg of purified ovalbumin were used to stimulate triplicate sets of PBL cultures to ensure the validity of lymphocyte stimulation by intact and denatured virus glycoproteins and infectious virus.

In another experiment, 5μl each of Mabs clones H1, H3, H4 and H6 against M63 and 5μl each of treated polyclonal antisera against F79 and A/Eq/Newmarket/76 (NMK76) and normal goat serum were added to triplicate cultues of PBL. The cells were then stimulated with 100μg of Ib4 virus glycoproteins and lymphocyte proliferation assays were carried out.

(C) The role of memory B and T cells in the protection of previously exposed (immunised) animals against challenge with infectious virus

Two of the three test animals previously given iv glycoprotein injections and later challenged with infectious virus (they were now aged 15 and 17 months respectively about 11 months after the previous challenge) and an adult influenza naive donkey were used as test animals while another adult donkey served as unchallenged control. Sera from the 4 animals were tested for NT antibodies against influenza virus. Titers of <80 were observed in the 2 previously challenged animals and <10 in the 2 influenza naive donkeys. The three test animals were then infected with 5ml of AF virus of Ib6 at $10^9 EID_{50}$ intranasally and observed for 28 days. Serum was collected from each animal at 7 and 21 days post challenge and serial 2-fold dilutions of the sera and a normal goat serum were reacted in NT tests with infectious AF of Ib6 virus.

3.3 RESULTS

3.3.1 Virus infectivity

3.3.1.1 Plaque assay

The plaques formed by the new virus isolates in the absence of trypsin were so indistinct that they could not be counted. In plates containing trypsin large visible plaques were seen and counted. Plaque titers were respectively $10^{8.64}$, $10^{7.93}$ and $10^{7.54}$ for Ib4, Ib6 and Ib9 (Table 5)

3.3.1.2 Virus titration in embryonated hen eggs.

The HA titers of each virus were inversely proportional to the dilution factors and were respective 8.51, 7.86 and 7.69 for the 3 virus isolates (Table 5).

The results of both assays indicate that the three viruses were infectious. The viruses grew well under the agar overlay containing trypsin to form large, visible and well defined plaques. Plaque efficiency was comparable to that of virus infectivity in fertile eggs as both PFU and EID_{50} values were very similar for the three isolates. It is possible that the trypsin-like proteolytic enzymes contained in the AF of fertile hen eggs enhanced virus infectivity.

Table 5 shows the PFU and EID_{50} titers and their ratios.

	Titers (log_{10}/ml)		PFU/EID_{50} ratios
Virus	PFU	EID_{50}	(log_{10})
A/Eq/Ibadan/4/91 (Ib4)	8.46	8.51	1:1.01
A/Eq/Ibadan/6/91 (Ib6)	7.93	7.86	1:1.01
A/Eq/Ibadan/9/93 (Ib9)	7.54	7.69	1:0.98

3.3.2 Virus mitogenicity and antigenicity

3.3.2.1 *In-vitro* mitogenicity

Fig. 13 shows the dose response curves of equine PBL against the virus glycoproteins of both subtypes of equine influenza A viruses. Maximum stimulation of PBL occured at concentrations between 50μg and 75μg in H3N8 viruses and between 75μg

and 100μg in the H7N7 virus. A plateau was also observed in lymphocyte responsiveness at concentrations between 50μg and 100μg in H3N8 viruses before the SI dropped slightly at 200μg in both virus subtypes. Lymphocyte stimulation was more marked in Ib4 virus, followed by Ib6 and M63 viruses which gave slightly similar responses while it was much less in Ib9 virus. On the other hand, P56 virus gave a steady increase in lymphocyte responsiveness beyond 100μg before dropping. Lymphocyte responsiveness was more pronounced with the positive control lectins while there was little or no cell proliferation in the PBS negative control cultures. The 96 hr cultures gave similar curves but with much less SI (data not shown).

The SI values from the parallel lymphocyte counts determined with Haemalog D were essentially similar to those of cell proliferation assays which thus validate the assays.

3.3.2.2 *In-vivo* mitogenicity, antigenicity and immunogenicity

(i) *In-vivo* PBL proliferation

Lymphocyte responsiveness to *in-vivo* stimulation by the virus glycoproteins is shown in Fig. 14. The glycoproteins induced PBL proliferation from 72 hr up to 6 weeks post iv injection. Maximum proliferation was observed at 72 hr with SI of 9.16, 7.88 and 8.62 for Ib6, M63 and P56 respectively. The SI was slightly less for each virus at 1 week post injection with P56 giving a better responsiveness than Ib6 and M63 viruses. However, lymphocyte responsiveness decreased significantly by about 45-66% in all cases from 2-6 weeks but there was no significant difference in individual responsiveness during this period.

Fig. 13. Dose response curves of equine peripheral blood lynphocytes (PBL) cultures at 72 hrs.

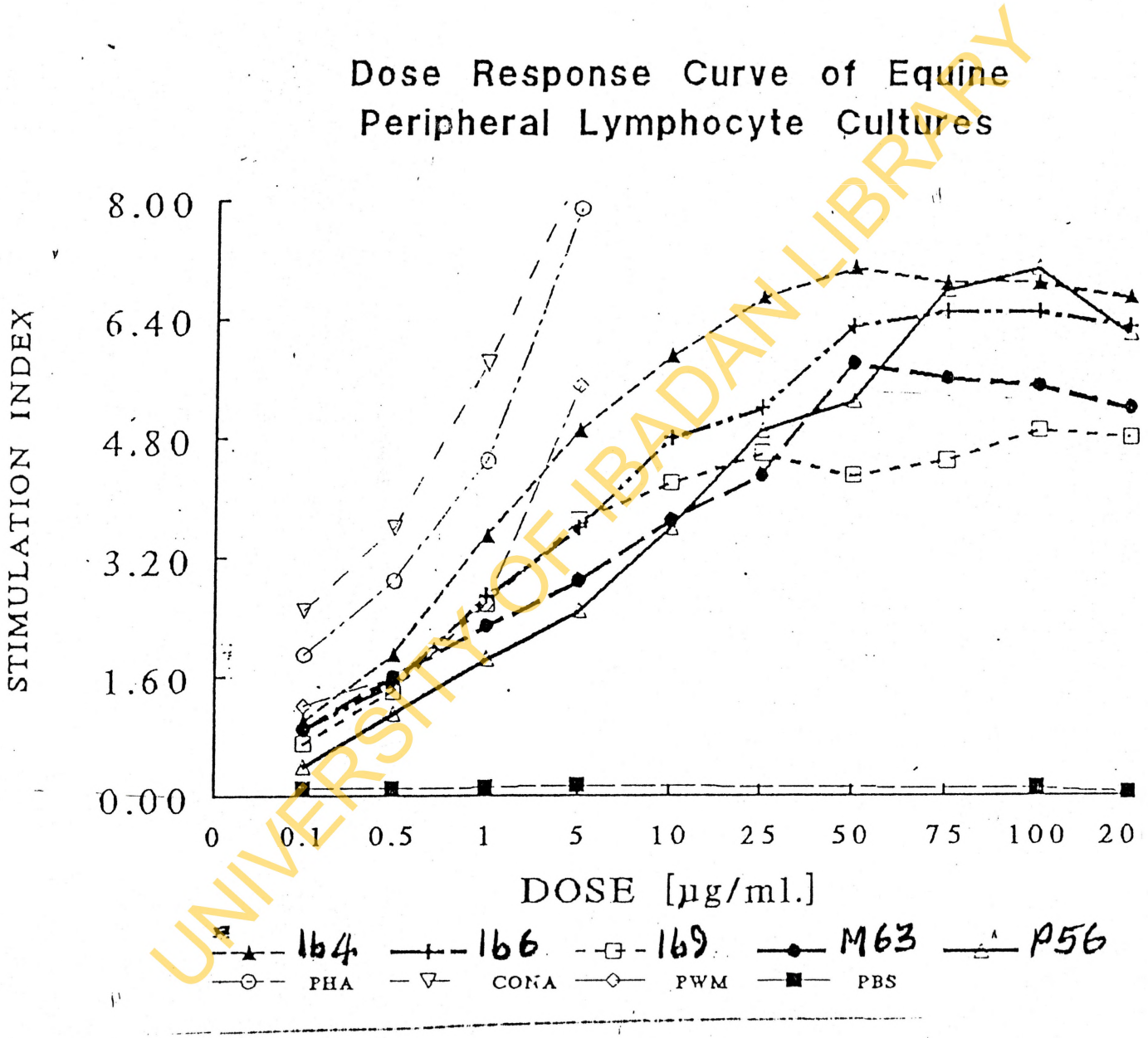
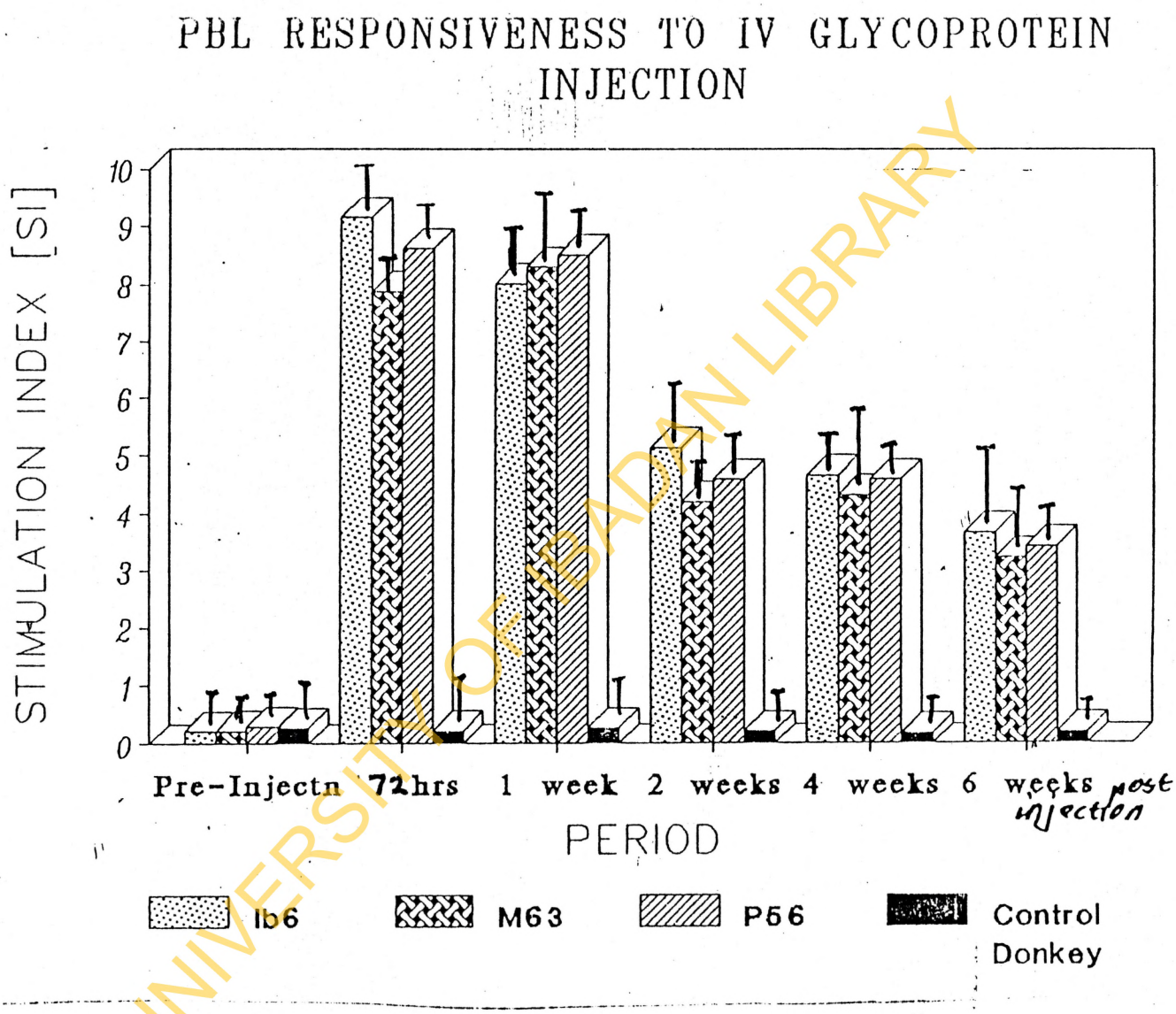


Fig. 14. PBL responsiveness to intravenous glycoprotein injection.



(ii) Serology

HI and NT antibodies were demonstrated in the sera of animals collected at 72 hr to 12 weeks post iv glycoprotein injection. In the NT tests CPE was observed in the wells containing the negative control serum and very weak dilutions of the test and positive control sera. HI and NT antibody titers are shown in Tables 6 and 7 respectively. Precipitin lines were also obseved in sera collected between 4-12 weeks in CIEP and immunodiffusion tests between the antigen wells and the positive control and test sera wells but not between antigen and negative control wells.

(iii) Experimental infection

The animals previously injected with virus glycoproteins and unchallenged control animal did not show any overt clinical signs of influenza infection except a slight pyrexia in the test animals which thus simulated previous immunisation while the previously uninjected control donkey had a rectal temperature of 39.5°C on the fifth day post infection followed by congestion of mucous membranes and bilateral serous oculo-

Table 6. HI titers of influenza virus AF antigens against post intravenous glycoprotein injection sera.

Viruses	Positive control rabbit anti-serum to		Pre-injection serum	Post iv glycoprotein injection serum test sera collected at						Negative control serum
	P56	F79		72 hrs	1 Wk	2 Wks	4 Wks	6 Wks	12 Wks	
A/Eq/ Ibadan/6 /91 (Ib6) H3N8 Yearing colt	10	1280	<10	20	40	320	640	1280	1280	<10
A/Eq/ Miami/ 63 M63 H3N8 6 month old colt foal	10	640	<10	10	20	160	160	640	1280	<10
A/Eq/ Praque/ 56 H7N7 4 month old donkey foal	2560	10	<10	10	20	160	320	640	640	<10
A/Eq/ Ibadan/ 4/91 Ib4 H3N8 Conrol antigen	10	1280	<10	10	20	320	160	1280	1280	<10

Table 7. Neutralisation titers of influenza virus AF antigens reacted with 10-fold dilutions of post intravenous glycoprotein injection sera.

Viruses	Positive control rabbit anti-serum to		Pre-injection serum	Post iv glycoprotein injection serum test sera collected at						Negative control serum
	P56	F79		72 hrs	1 Wk	2 Wks	4 Wks	6 Wks	12 Wks	
A/Eq/ Ibadan/6 /91 (Ib6) H3N8 Yearing colt	1	160	< 1	8	32	16	128	512	512	< 1
A/Eq/ Miami/ 63 M63 H3N8 6 month old colt foal	1	320	< 1	8	16	16	64	512	512	< 1
A/Eq/ Praque/ 56 H7N7 4 month old donkey foal	320	1	< 1	4	64	32	256	512	512	< 1
A/Eq/ Ibadan/ 4/91 Ib4 H3N8 Conrol antigen	1	160	< 1	8	8	32	128	256	512	< 1

nasal discharges on the sixth and seventh days. The animal was dull and anorectic and coughed occassionally from the eighth day for about 3 days after which the signs finally abated. Paired sera collected on days 5 and 14 post infection showed rising HI titers of 20 and 160 respectively in this animal while there was little or no difference in the titers of the two previously injected animals. However, no virus was recovered from any of the animals.

3.3.3 Virus immunogenicity

(A) Characterisation of the proliferating lymphocytes

Passage of PBL over nylon wool thrice depleted B-cells to presumably <1.4% of total lymphocyte populations. Lymphocyte proliferation was induced in T-cell enriched cultures with SI of 5.8±1.6 comparable to SI of 6.1±0.2 in undepleted PBL cultures. This implies that equine influenza virus glycoproteins are both B and T-cell mitogens.

(B) Induction of equine influenza virus glycoprotein mitogenicity and immunogenicity

Infectious virus, intact and denatured glycoproteins induced lymphocyte proliferation *in-vitro* and *in-vivo* to similar extents as indicated by SI shown in Table 8. There was no significant differences in SI obtained in in-vitro and in-vivo cultures. Lymphocyte proliferation was inhibited by Mabs and polyclonal antisera added to stimulated cultures while proliferation was observed in the well to which normal goat serum was added. The implications of these results are that the mitogenic and immunogenic activities of equine influenza virus glycoproteins appear to be induced by direct binding of the protein antigens to receptors on the cell surface.

Table 8: *In-vitro* and *in-vivo* stimulation indicies of infectious virus, intact and denatured virus glycoproteins.

	Stimulation indicies	
Stimulation agents	*In-vitro*	*In-vivo*
Infectious virus	5.2±1.3*	4.7±0.6
Intact glycoprotein	6.4±0.8	6.2±1.7
Denatured glycoprotein	6.3±1.1	6.8±0.54

* Standard deviation.

(C) The role of B and T memory cells in the protection of previously exposed (immunised) animals against challenge with infectious influenza virus

The sera obtained at 7 and 21 days post challenge from the 3 test animals neutralised AF virus. The 3 animals did not show any clinical signs during the 28 days of observation. The serum of unchallenged control animal failed to neutralise AF virus and showed extensive CPE after 48 hr of incubation. NT titers at 7 days post challenge were 16-640 in the 3 animals but at 21 days post challenge, titers in the 2 previously injected (immunised) animals were 320-1024 while they were 160-512 in the previously uninjected but challenged donkey. The demonstration of high NT titers in the 2 previously injected (immunised) animals probably implies that the memory B and T cells were activated.

3.4 DISCUSSION

The enhanced growth of the new virus isolates in the presence of trypsin, a proteolytic enzyme, is a reflection of the environment in which influenza virus survives in nature as the fluid bathing the alveoli and lower respiratory tracts in infected animals contain proteolytic enzymes chiefly derived from lysosomes of alveolar macrophages (Harries *et al.*, 1975). Acquisition of infectivity by cleavage of the HA into HA1 and HA2 enhanced by proteolytic enzymes is thus a part of the mechnism of influenza virus maturation *in-vivo*. (Walker *et al.*, 1992). Allantoic fluid of embryonated hen eggs also contains proteolytic enzymes which make the allantoic membrane cells universally susceptible to all influenza virus strains and are thus used for primary isolation and determination of virus infectivity. It appears that the three virus isolates were infectious

enough as observed in plaque assay and egg titration of the viruses and as shown in experimental infections of susceptible animals used in this study.

It is known that carbohydrate side chains can potentially modulate the biological activity of influenza virus HA glycoprotein. In both H1 and H3 HAs, addition of a carbohydrate moiety can block antibody binding at a site to which in the absence of the carbohydrate, it would be capable of binding (Wiley and Skehel, 1987). The H1 and H3 influenza virus subtypes are examples of viruses that use this mechanism to avoid antigenic surveillance in human hosts (Caton *et al.*, 1982). It is probable that an additional glycosylation site resulting in the addition of a carbohydrate moiety has occured in the present H3 Ibadan equine influenza virus isolates with the consequent escape of antibody binding and neutralisation and increased infectivity which resulted in overt clinical infection of previously unexposed experimental animals used in this study (See pages 182 and 198).. Gibson *et al.*, (1992) have reported the occurence of two new glycosylation sites on equine H7 HAs, one of which is close to the HA1 and HA2 proteolytic processing site and which could modulate virus antigenicity. Similarly, the presence of a carbohydrate close to the HA cleavage site in H5 avian influenza virus HAs has been shown to dramatically influence virus infectivity (Bosch *et al.*, 1981). Complete nucleotide and amino acid sequence and antigenic structural analysis should elucidate this probability in the new virus isolates.

The results of *in-vitro* mitogenicity are in accord with previous findings in human and mice (Hurwitz *et al*, 1985; Hackett *et al*, 1991) and support the hypothesis that equine

influenza virus glycoproteins could induce mitogenic lymphocyte proliferation of equine PBL. The HA may be more responsible although the NA is also a target of antiviral immune responses (Reay *et al.*, 1989). The use of PBL from a previously unexposed animal precludes initial priming of the cells which thus gave a primary response unrelated to any memory cells in the first instance. It was thus possible to subsequently use this as a fruitful model in the study of overall immunological response of equines to influenza virus infection. The *in-vivo* responses of PBL to virus glycoprotein stimulation also demonstrate that the glycoproteins are also mitogenic and immunogenic *in-vivo*. The ability to record good lymphocyte proliferation and to demonstrate HI, NT and precipitating antibodies in the sera of test animals from 72 hr up to 12 weeks post iv glycoprotein injection support this and validates the hypothesis. The animals were also protected against experimental infection 6 months after being given the virus glycoproteins which thus simulated previous immunisation while the previously uninjected animal became sick. It is known that influenza infection or immunisation is followed by development of HI and NT antibodies which inhibit virus release and neutralise the virus *in-vivo*. Anti-HA and anti-NA antibodies are thus very important active factors in immunity to influenza virus infection. Such antibodies have been demonstrated for a considerable length of time in the present study thus confirming that primary exposure to influenza virus is characterised by a sustained presence of antibodies in peripheral blood circulation (Hannant *et al.*, 1988; Richards *et al.*, 1992). It is probable that NT antibodies mediate immediate infection while the memory cells are responsible for long term immunity. It is therefore reasonable to

suggest that the antibodies demonstrated in the present study conferred a long term protection on the susceptible animals since the virus glycoproteins are capable of activating both humoral and cellular immune responses. The protection of the animals at 6 months post iv glycoprotein injection and production of immunoglobulins about 11 months post glycoprotein injection followed by challenge with infectious virus support this view.

Cellular immune response comprises a complex interaction between different cell types and molecules and T-cell induction requires antigen processing, presentation and recognition by T-cell receptors (Beerzosky *et al.*, 1988). T-cells recognise antigens/mitogens through their association with nascent major histocompartibility (MHC) chains (Lanzavecchia, 1990) and are thus activated. Although it is difficult to relate the amount of virus glycoproteins injected to the amount recognised by the T-cells, it was quite possible to demonstrate the presentation to and activation of T-cells and B-cells by the glycoproteins as the PBL responses have been quantitatively measured and antibody responses have been demonstrated in this study.

Stimulation of B and T-cells by the glycoproteins demonstrate that equine influenza virus glycoproteins are both B and T-cell mitogens. This is in accord with previous obervation with hunan viruses by Armstrong *et al.* (1981) but contrasts with that of Anders *et al.*, (1984) who reported influenza HAs as T-cell independent B-cell mitogens. Passage of lymphocytes over nylon wool twice is expected to reduce B-cell population to <1.4% (Anders *et al.*, 1984). In the present study, PBL were passed over nylon wool thrice to ensure complete removal of B-cells but similar SI were obtained for T-cell enriched and undepleted cultures thus leading to the conclusion that the virus glycoproteins were B and

T-cell mitogens. Hurwitz *et al.*, (1985) also demonstrated significant proliferation of purified T-cell populations in response to isolated HA, NA, M and NP proteins of influenza viruses. It is likely that Anders *et al.*, (1984) did not completely deplete B-cells in their study. A limitation of the present study however was the inability to further determine the T-cell subsets and their linages as well as the quantity of lymphokines produced by them due to lack of facilities to undertake such studies apart from being outside the scope of the present study.

Although, it has been reported earlier that induction of influenza virus glycoprotein mitogenicity is by activation of classes I and II MHC restricted T-cells (Hurwitz *et al.*, 1985; Reay *et al.*, 1989; Mycoccka and Hackett, 1990), in the present study induction of equine influenza virus glycoprotein mitogenicity and immunogenicity was observed to be by direct binding of the virus envelope protein antigens to receptors on lymphocytes as demonstrated by the inhibition of lymphocyte proliferation by the Mabs and polyclonal antisera used which must have prevented binding of the glycoproteins to the lymphocytes. Similar levels of lymphocyte stimulation were also observed when infectious virus, intact and denatured glycoproteins were used as stimulants. This suggests that influenza virus-induced T-cell responses do not require the native conformation of the virus per se. The interaction between B and T-cells and the resultant products are crucial to the animal's immune response. Cognate interaction involves conjugation of one B-cell and one T cell (Sanders *et al.*, 1988) and leads to activation of T-cells which in turn can regulate cellular and humoral immune response either by further cell-cell contact or by release of

lymphokines. The resting B-cells can thus be stimulated to proliferate and produce antibodies whose isotype switch from IgM to IgG, IgA and IgE could be mediated by T-cell lymphokines.

The results above also demonstrate that previously immunised animals remain protected for a long time (almost a year). This protection could be due to high titers of HI and NT antobodies in the serum and possibly some locally produced mucosal antibodies as earlierb observed by Wright *et al.* (1983). The higher antibody titers observed in the sera of previously exposed animals compared to the naive animal indicate that memory cells were induced with the resultant production of a large amount of immunoglobulins. The implication of this is that the protection engendered by these antibodies is related to activation of memory B and T-cells although mucosal immunity may have played some role. The results also justify earlier speculations in an equine model on the possible use of influenza virus glycoproteins as denatured or proteolytic fragments in the study of the primary response of equines to infection or even as immunogens for protecting valuable animals taking into consideration the effects of carriers and adjuvants used in conventional vaccines since they enhance or suppress the animal's immune response. These results have in a way contributed significantly to the understading of the overall immunological response of equines to influenza virus infection.

CHAPTER 4

MOLECULAR CHARACTERISATION OF INFLUENZA VIRUSES ISOLATED FROM EQUINES IN NIGERIA

4.1 INTRODUCTION

Influenza A viruses can infect a wide variety of avian and mammalian hosts and mixed infection with different influenza virus strains can lead to reassortment of genomic RNA segments (Laver and Webster, 1973; Webster *et al.*, 1992) which could result in the origin of new influenza pandemics. Influenza virus subtypes infecting mammalian and avian hosts demonstrate varying degrees of antigenic drift and genetic shift. Minor antigenic drift in the HA of equine H7N7 virus isolates has been detected (Burrows and Denya, 1982) while Daniels *et al* (1985) and Kawaoka *et al.* (1989a) and Berg *et al.* (1990) have also reported significant antigenic drift in equine H3 subtype HA. Analysis of the nucleotide sequences of each of the 8 influenza A virus gene segments may provide valuable information about the evolutionary relationship among these viruses isolated from different hosts in diverse geographical locations around the world. The evolutionary pathways of these genes have now been established (Webster *et al.*, 1992, Fig. 7 in chapter two) but new strains of influenza A virus continue to emerge in humans, lower animals and birds (Webster and Kawaoka, 1994).

The degree of antigenic variation within equine influenza viruses is of considerable interest in order to determine their origin, evolution and genetic relatedness in view of possible reassortment of these gene segments to produce hybrid strains or new viruses and

the possible transfer of whole or part of influenza virus genome of avian origin into a mammalian host as recently reported in horses in Northeast China (Guo *et al.*, 1992). Rapid evolution of influenza A viruses in man and animals has continued since recorded medical history and is dependent on periodic introduction of gene segments or entire viruses from the avian influenza virus gene pool. This continuing evolution is most prominent in the surface glycoproteins (HA and NA) of influenza A viruses but also occurs in each of the 8 gene segments of both type A and type B viruses.

Phylogenetic analysis of the nucleotide sequence of the HA gene of equine-2 influenza viruses has been carried out and distinct conclusions relating to the epidemiology and evolution have been drawn. An analysis carried out by Endo *et al* (1992) suggested that two main evolutionary lineages of the viruses were present but this suggestion was in contrast to the conclusion earlier presented by Kawaoka *et al* (1989a) who adduced that only a single evolutionary lineage could be described. The most recent analysis of equine H3 viruses, which includes analysis of additional viruses isolated between 1989 and 1991, concluded that there was a change in the 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 (Oxburg *et al.*, 1994). In total contrast, the viruses that were isolated in China in 1989 and 1990 were shown to have a distant phylogenetic relationship to typical equine-2 HA genes and furthermore the genes which encode the internal proteins in these viruses were derived from virus strains associated with birds rather horses (Guo *et al.* 1992) but these viruses had been superceded by equine H3N8

viruses during 1993-1994 epidemic of equine influenza in China (Shortridge *et al.* 1995, Gu *et al.* 1995). In the light of this background information an analysis of the Nigerian equine influenza virus isolates was been undertaken. In this study the complete nucleotide sequence of the HA gene of these Ibadan viruses is presented and limited sequence analysis of each of the genes encoding the internal proteins was also carried out.

4.2 MATERIALS AND METHODS

4.2.1 Phenol extraction of virion RNA

To 500µl of each of the purified virus isolates resuspended in Tris-saline in a 1.5ml microfuge tube were added 25µl of 0.5M EDTA pH 8.0 to a final concentration of 5mM and 25µl of 10% SDS to a final concentration of 0.5%. The tubes were vortexed to mix the contents and were placed under a running hot tap water or incubated at 60°C in a water bath for 1 minute to dissolve. To this solution was added 50µl of 0.5M sodium acetate pH 5.0 to a final concentration of 50mM, followed by an equal volume of water saturated phenol previously stored at 4°C. The tubes were thoroughly vortexed to give a single phase and were centrifuged at 14,000 rpm in a bench top Eppendorf microfuge for 10 min at 10°C. The top equaous phase containing the RNA was transfered into another 1.5ml microfuge tube with a pasteur pipet, care being taken at the interphase. An equal volume of water saturated phenol was added and centrifuged as before. The upper aqueous phase was transfered into fresh microfuge tubes and exracted with two volumes of diethyl ether, the upper ether phase being discarded each time. The RNA in the lower phase was divided into two fresh tubes and precipitated either at -20°C overnight or at -70°C for 30-60 min

by addition of 1:20 volume of 3M sodium acetate pH 5.2 to a final concentration of 0.3M and 3 volumes of ethanol (i.e. 250µl of 3M sodium acetate and 750µl of ethanol) followed by centrifugation at 14,000 rpm for 10-15 min in a microcentrifuge at room temperature (rt) of 22-25°C or 4°C. The pellets obtained were resuspended in 100µl sterile double distilled water (sdw) after thoroughly draining the supernatant with a drawn-out Pasteur pipett. The RNA was then reprecipitated by addition of about one third volume of 10M ammonium acetate and three volumes of ethanol, mixed and stored at -70°C or dry ice (frozen CO_2) for 30 min or at -20°C for 2 hours followed by centrifugation in a microfuge for 10 min. The supernatant was thoroughly drained and the pellet rinsed with ice cold ethanol, completely drained using drawn-out pasteur pipet and the pellet dried in a speedvac (UniSci Univap) for 10 min. The RNA pellets for each virus isolate were then resuspended in 100µl sdw in 1.5ml microfuge tubes.

The concentration of each virion RNA (vRNA) extracted was determined with a Shimadzu double beam ultra violet (UV) spectrophotometer model 150-02 or a Philip scan UV spectrophotometer. The vRNA was stored at -20°C until required.

4.2.2 Examination of extracted vRNA

Following extraction, the vRNA of each segment was examined and the molecular weights (M_r) were estimated by means of 1% agarose gel electrophoresis under denaturing conditions with 2.2 M formamide using Hind III-digested phage DNA or ribosomal RNAs as size marker. One percent agarose was prepared by dissolving 1gm of agarose in 100ml 1X Tris-borate-EDTA (TBE) in a Thompson microwave oven set at 2 for 4-5 min. The gel

was then poured into a minigel apparatus (ScotLab) after cooling at room temperature to about 35°C and allowed to set. 1X TBE was added to cover the gel and 3µl of glycerol dye was added to 7-10µl of each vRNA, mixed on the back of Whatman sealing film and loaded into the wells of the gel which was then run with a Bromma LKD constant electrofocusing power supply at a constant voltage of 75 volts for 45-60 min. After this, 15µl of 10mg/ml ethidium bromide solution was added to the TBE running buffer to stain the RNA bands for 5-10 min after which the bands were visualised with transmitted shortwave UV light and photographed with a Polaroid camera.

4.2.3 Preparation of infected cell RNA from confluent monolayer cells

Infected cell (i.c.) RNA was extracted from cells as described by Hay *et al.* (1977) with some modifications. Monolayer cells in 15ml tissue culture dishes were first rinsed with Ca^{++} and Mg^{++} free PBS. They were then inoculated with 5ml infectious allantoic fluid per dish from the working stock at a multiplicity of infection (m.o.i.) of 10 using two dishes for each virus isolate and incubated at rt for 30 min. The dishes were rocked every 5-10 min. After incubation the inoculum was removed and the cells were rinsed thrice with warm PBS or maintenance menimum essential medium (MEM). The cells were covered with 15-20ml of MEM containing 5-10% fetal calf serum (fcs) and incubated at 37°C + 5% CO_2 for about 6 hours.

After incubation, the medium was removed and the cells were rinsed with ice cold Tris-saline. Four mililiters of lyse buffer (50mM sodium acetate pH 5.0, 2.5mM EDTA pH 8.0 and 0.5% SDS) was added to each dish, gently mixed and the cells were removed with

a sterile rubber policeman into 50ml Falcon graduated centrifuge tubes for each virus, care being taken to avoid sharing the nucleic acids. An equal volume (8ml) of cold water saturated phenol was added, the two phases were gently mixed in a 65°C water bath or under hot tap water until a single phase was observed. The tubes were then centrifuged at 2,500 rpm at 10-15°C for 10 min in a Central 4R general purpose rotor. The upper equaous phase was carefully removed with a pasteur pipet into fresh tubes and re-extracted with an equal volume of water saturated phenol as above. The upper equaous phase was then transfered into sterile 30ml Corex tubes and i.c. RNA was precipitated overnight at -20°C by adding 3M sodium acetate pH 5.2 to a final concentration of 0.3M and three volumes of ethanol followed by centrifugation in a Sorvall centrifuge at 12,000 rpm for 20 min at 4°C in SS34 rotor. The pellets obtained were completely drained using sterile tissue paper and resuspended in 300µl sdw. The suspension was then transfered into 1.5ml microfuge tubes and the RNA was re-precipitated with 75µl of 10M ammonium acetate and 750µl of ethanol followed by storage at -20°C for 30-60 min or at -70°C or dry ice for 15 min and centrifugation in a microcentrifuge for 10 min at 4°C or room temperature. The pellets were completely drained of ethanol with drawn-out pipet and resuspended in 500µl sdw with thorough vortexing. When the pellets were difficult to dissolve and resuspend, the tubes were placed in a 60°C water bath for 2-5 min followed by thorough vortexing or on ice for 5-10 min followed by vortexing.

The concentration of i.c. RNA was determined by adding 5µl of extracted RNA to 500µl sdw in fresh 1.5ml microfuge tubes and measuring the absorbance at 260nm wavelength in a Philip scan or Shimadzu UV spectrophotometer. The contentration was

calculated from the formula.

$$\frac{A_{260} \times \text{dilution factor of } 100}{20} = \text{concentration in mg/ml}$$

Fifty µl of each i.c. RNA at 1µg/µl was prepared and stored at -20°C until required while the remaining RNAs were stored at -20°C in 100µl aliquots. All the materials used were RNAse free.

4.2.4 Synthesis, purification, deprotection and examination of oligonucleotide primers

All the oligonucleotide primers used were synthesised on an Applied Biosystems 381A DNA synthesiser. After synthesis the primers were either deprotected or gel purified.

4.2.4.1 Deprotection

About 3ml of concentrated ammonia solution were aliquoted into a small sterile bijou bottle in a sterile laminal flow hood and 0.5ml of ammonia was drawn into the barrel of a 1ml syringe which was attached to one end of the oligonucleotide mini-column. Air was drawn up to 0.5ml mark into a second 1ml syringe which was also attached to the other end of the mini-column. The air was pushed into the mini-column followed by about 0.4ml ammonia from the other syringe ensuring that there were no air bubbles while the plunger of the other syringe containing air was drawn out up to 0.8ml mark. The mini-column was then rotated through a 360° to mix the ammonia and oligonucleotide thoroughly. The attached mini-column and syringes were left at room temperature in the hood for 30 min after which the ammonia solution/oligonucleotide

mixture was pushed through the mini-column into a 2ml Sarsdect screw cap tube with skirt. The procedure was repeated twice more to give a total volume of 1.5ml. The tube was then incubated in a 60°C water bath for at least 6 hours and vacuum-dried in a speedvac for about 5-8 hours. The pellet left in the tube was resuspended in 1.5ml sdw or Tris-EDTA (TE) buffer.

The concentration of deprotected oligonucleotide primers was determined by adding 5µl to 500µl sdw and measuring the absorbance at 260nm as above.

4.2.4.2 Examination and gel purification

Twenty percent acrylamide gel was prepared in 40cm x 20cm glass gel plates with the comb inverted to form a continuous trough without wells. To 150µl of oligonucleotide primer in 0.5ml Eppendorf microfuge tube was added 150µl of formamide plus bromophenol blue without xylene cyanol. The contents were mixed, incubated at 60°C for 10 min and loaded on the gel which was run at 36 watts and 1,600 volts for 2-3 hours or until the bromophenol blue front was mid-way down the gel. The top gel plate was carefully removed, the gel slab was covered on both sides with cling film, placed on a thin layer chromatography (TLC) sheet and visualised with transmitted UV light to observe the bands.

Each band was cut out with a sterile scapel blade and placed in a marked sterile plastic universal bottle. The cling film was gently removed, 4ml sdw was added and the bottle was incubated in 68°C water bath for 30-60 min to extract the primer. The sdw was sucked out with a pasteur pipet into a fresh universal bottle and stored at 4° C overnight

while another 4ml sdw was added to the first bottle containing the gel and incubated at 37°C overnight. The eluates were filtered through a 0.2µl Millex millipore filter into a snap cap Falcon 2059 tube starting with the first eluate stored at 4°C overnight. To the filtrate was added an equal volume of butan-2-ol, mixed and centrifuged for about 1 min at 1,200 rpm in a bench top Central 4R or Denley general purpose centrifuge to give two phases. The top butan-2-ol phase was removed and discarded while an equal volume of fresh butan-2-ol was added to the lower phase, mixed and centrifuged as before. The process was repeated several times until about 500µl was left in the lower phase.

Sephadex G50 fine columns were prepared in two 5ml syringes using siliconised glass wool as a bed. Each column was washed through with about 10ml sdw using 500µl sdw at a time. After thorough washing, 500µl oligonucleotide extract was applied to the columns and about 500µl eluate was collected into marked 1.5ml microfuge tubes. The absorbance at 260nm of each eluate was measured on a UV spectrophotometer. Five peak readings were selected (the top three and one on either side of them) and the five tubes were dried in a speedvac. Alternatively, they were pooled and dried in a tube. From the optical density values, calculations were made using the top three readings to determine the volume of sdw with which to resuspend the dry oligonucleotide pellets. The calculated volume of sdw was then added to resuspend the pellets one after the other until all the pellets in the three tubes were completely dissolved in a tube. If the eluates were pooled, the calculated volume of sdw was used to resuspend the single pellet in one tube. This calculated volume is equal to 10µM of oligonucleotide primer.

Alternatively, 5µl of resuspended oligonucleotide primer was diluted in 500µl sdw

and the absorbance at 260nm was measured. The value was then used to calculate the concentration of the primer. Table 10 shows the list and concentration of all oligonucleotide primers used while their sequences are shown in Appendix F.

4.2.5 Reverse transcription of vRNA segments

This required oligonucleotide primers which are short lengths of DNA complementary to the RNA genome sequences of influenza A viruses. To generate copies of all the eight segments of an influenza virus, a primer corresponding to the 12 terminal nucleotides from the 3′ end of Rostock strain of influenza virus RNA was used. Alternatively the 13 terminal nucleotides at the 5′ end constituted the primer since these terminal nucleotides are conserved in all influenza A virus RNA segments. For a standard reverse transcription (RT) reaction, 0.7nmoles of the first strand primer (12 terminal nucleotides at the 3′ end) was phosphorylated with 1mM ATP using 40 units of T4 polynucleotide kinase (Epicenter) in 10X T4 polynucleotide kinase buffer (50mM Tris-HCl pH 7.5 at 25°C, 100mM $MgCl_2$, 50mM DTT, 1mM spermidine) in a 50µl reaction incubated at 37°C for 30 min followed by addition of 200µl of sdw to make a 1:50 dilution of the primer. All materials used were kept on ice.

For each of the virus isolate, 2-5µg of vRNA or i.c. RNA or 10µg of cRNA was added to about 8 picomoles (pmoles) of kinased first strand primer and sdw was added to a total volume of 10µl in 0.5ml microfuge tubes. The reaction tubes were boiled for 2 min and immediately placed on wet ice. A radioactively labelled reaction mix was made up in a separate tube containing 0.5mM dNTPs (dATP, dCTP, dGTP and dTTP), 10µCi α^{32}P-

dATP at 800Ci/mmol, 40 units of RNAsin (Promega), 5X RT buffer (350mM KCl, 250mM Tris-HCl pH 8.2, 40mM $MgCl_2$, 50mM DDT) to a final conentration of 50mM Tris-HCl, 75mM KCl, 3mM $MgCl_2$ and 10mM DTT, 16 units of AMV RTase enzyme and sterile RNAse free sdw to a final volume of 10µl per reaction tube. Ten µl of this reaction mix was then added to each primer-template tube. The reaction tubes were incubated in a 42°C water bath for 45-60 min. An equal volume of formamide dye containing xylene cyanol was then added to each reaction tube to stop the reaction and the samples were boiled for 2 min before being loaded on a 4% polycarylamide gel in 20cm x 20cm glass gel plates. Ribosomal RNAs were run with the gel as M_r markers to determine the M_r of each virus RNA segment.

4.2.6 Polyacrylamide gel electrophoresis of vRNA segments

Four percent acrylamide gels were cast in 20cm x 20cm glass gel plates separated with 4-5mm spacers and 20 tooth combs and allowed to set after polymerisation with 0.1% APS and 0.1% TEMED as in chapter two. The gels were pre-run for at least 20-30 min and samples of the RT reactions were boiled for 2 min prior to loading. The gels were run in 1X TBE running buffer (100ml 10X TBE, 900ml deionised water) at 30 mAmps and 500 volts for about 90 min. The gels were fixed in 10% methanol and 10% glacial acetic acid for 30 min and vacuum dried as described in chapter 2 above. Radioactivity in wet and dried gels was monitored with the Radman monitor.

4.2.7 Generation of cDNA through polymerase chain reaction (PCR) amplification

To generate cDNA, the first and second strands of the cDNA were first synthesised from vRNA or i.c. RNAs of each virus isolate before the PCR was perfomed. For the PCR, primers corresponding to the terminal 12 nucleotides at the 3' end of Rostock vRNA with an Xho 1 restriction site (first strand primer) and 13 terminal nucleotides at the 5' end of Rostock vRNA with a Not 1 restriction site (second strand primer) were used. First strand synthesis was basically a standard RT reaction similar to that in reverse transcription of virus RNA sgments above except that the reaction volume was 25µl and atimes, the primer-template was not boiled and placed on wet ice and the primer was not labelled with a radio-isotope. Five µl of this first strand synthesis was used as template in the second strand synthesis. To this template was added about 20 pmoles of a phosphorylated reverse second strand primer, 5X Dupont (TSP) RT buffer to a final concentration of 50mM Tris-HCl pH 9.5, 1.5mM $MgCl_2$, 20mM $(NH_4)_2SO_4$. This was heated at 95°C for 4 min in a water bath or thermal cycler to denature the template before adding 0.2mM dNTPs and 5 units of type II thermophilic DNA polymerase (TSP by Cambio). Alternatively, 10X Taq DNA polymerase buffer was added to a final concentration of 10µM Tris-HCl pH 9.5, 1.5mM $MgCl_2$ and 50mM KCl in place of TSP buffer with 5 units of Taq DNA polymerase enzyme (Boerhinger Mannheim) in place of TSP DNA polymerase. The reaction mix was then incubated at 60°C for 20 min and stored on ice until required.

For the PCR amplification of cDNA formed, 10µl of the second strand synthesis was used in separate reaction tubes suitable for the thermal cycler being used. To this were

added 5X TSP or 10X Taq buffer in double the concentration of that used for second strand synthesis, followed by addition of first and second strand primers in double the volumes used in first and second strand synthesis to a final concentration of 480nmoles and sterile RNAse free sdw to a final volume of 100µl.

The reaction mix was then denatured at 95°C for 4 min and hybridised at 40°C for 2 min followed by addition of 0.2mM dNTPs and 2.5-5 units of TSP or Taq DNA polymerase enzyme to each reaction tube which was briefly spun in a microcentrifuge to mix the contents which were then overlaid with 80µl of mineral oil or liquid paraffin to prevent evaporation. The reaction tubes were placed on the programmable thermal cycler set up with the following programs:60°C for 4 min to polymerise, 92°C for 2 mins to denature, 40°C for 2 mins to hybridise and 65°C for 20 min to extend, the process being repeated for 20-25 cycles. On completion, the mineral oil overlay was carefully extracted with diethyl ether and a 5µl aliquots of the PCR products were checked on a 1% agarose stained with ethidium bromide as done for extracted vRNA above. The remaining PCR products were purified with the Magic Preps™ DNA purification system (Promega).

Attempts were made to detect virus RNAs/cDNAs from the clinical samples directly through PCR amplification of 2-5µl of the samples using first and second strand primers in a PCR reaction.

4.2.8 Purification of (cDNA) PCR products

The PCR products were purified by aliquoting 100µl of direct purification buffer (50mM KCl, 10mM Tris-HCl pH 8.8, 1.5mM $MgCl_2$, 0.1% Triton X-100) into a 12 x

75mm polypropylene Falcon tube for each PCR product and adding 50-80µl of PCR products, mixing the tube contents followed by adding 1ml magic PCR Preps resin and brief vortexing thrice over a period of 1 min. The DNA purification resin containing the bound cDNA was gently pipeted into the barrel of a 2ml syringe to which was attached a mini-column was the slurry produced was gently pushed into the mini-column with the syringe plunger. The mini-column was washed with 2ml of 80% isopropanol and then transfered to a 1.5ml microfuge tube which was centrifuged at 14,000 rpm in a microcentrfuge for 20 seconds to dry. The mini-column was left at room temperature for 10-15 min to allow the isopropanol to evaporate before being transfered to a fresh microfuge tube. The bound cDNA was eluted with 50µl of TE buffer (10mM Tris-HCl pH 7.5, 1mM EDTA pH 8.0) added to the mini-column which was left at room temperature for 1-2 min before centrifuging for 20 seconds at 14,000 rpm. The mini-column was then removed and discarded while 5µl of eluted purified cDNA left in the tube was checked on 1% agarose. The purified cDNAs (PCR products) were stored in 10µl aliquots at -20°C until required.

4.2.9 Estimation of RNA, cDNA and oligonucleotide primer concentrations

All samples were diluted 1:100 with sterile RNAse free sdw which was also used as a blank. The absorbance at 230-290 nm was determined with a Shimadzu or Philip scan UV spectrophotometer using fixed or scan Lamda. The ratio of A_{260} over A_{280} which is approximately 2 (1.7-2) gives a good quality RNA while the ratio of A_{260} over A_{230} which is greater than 2 indicates that there is no contamination by the extraction and precipitating

reagents. The concentrations of RNAs and cDNAs were calculated from the formula given above while those of oligonucleotide primers were calculated from

$$\frac{A_{260} \times \text{dilution factor of } 100 \times 50}{1000} \times \frac{10^6}{M_r \text{ of primer}}$$

M_r of each primer = 300 x length of primer since each nucleotide has M_r of 300. Concentration of each primer was expressed in nmoles/ml = pmoles/µl.

4.2.10 Cloning of cDNA in plasmid vector

In order to compare nucleotide sequences and sequencing efficiency and to preclude sequencing of PCR artifacts or contaminants, full length copies of cDNA from each of the virus isolate were cloned in plasmid vector. Two independently derived clones of each gene were then sequenced to identify and resolve any cloning artifacts. cDNA was generated through PCR as described above. The TA Cloning™ System (Invitrogen) was used for direct insertion of the cDNA of each gene segment into the plasmid vector. Fig. 16 shows the resriction map and sequence of the cloning vector used in this study.

4.2.10.1 Cloning procedure

The absorbance at 260nm of the cDNA of each virus RNA segment was determined with a Philip scan spectrophotometer. All the materials used were kept on ice. The TA Cloning™ vector (3.9 kb PCR™ vector from Invitrogen) vial was briefly spun and resuspended in 8.8µl of TE buffer to a final concentration of 25ng/µl.

4.2.10.1.1 Ligation: Into each of 4 marked 0.5ml microfuge tubes were pipeted 1µl of resuspended vector to which was added 1µl of 10X ligation buffer (50mM Tris-HCl pH 8.0, 8mM $MgCl_2$, 5mM DTT, 1mM ATP). Inserts (2µl each of cDNA of the three virus isolates, A/eq/Ibadan/4/91, A/Eq/Ibadan/6/91 and A/Eq/Ibadan/9/91) were added to correspondingly marked tubes while the fourth tube served as control and 5µl sdw was added to each of cDNA tubes while 7µl sdw was added to the control tube followed by 5 units of T4 DNA ligase to give a total volume of 10µl of reactants. The tubes were incubated overnight in a 12°C water bath placed in a 4°C cold room. The ligation mixes were then stored on ice for immediate use or at -20°C until required for transformation.

Fig. 15. PCR™II cloning vector nucleotide sequence.

M13 Reverse Primer

```
CAG GAA ACA GCT ATG AC C ATG ATT ACG CCA AGC T
GTC CTT TGT CGA TAC TG G TAC TAA TGC GGT TCG A
```

Sp6 Promoter

```
AT TTA GGT GAC ACT ATA GAA
TA AAT CCA CTG TGA TAT CTT
```

```
                NsiI        HindIII    KpnI       SacI                SpeI          XmaIII
TAC TCA AGC TAT GCA TCA AGC TTG GTA CCG AGC TCG GAT CCA CTA GTA ACG GCC
ATG AGT TCG ATA CGT AGT TCG AAC CAT GGC TCG AGC CTA GGT GAT CAT TGC CGG
```

```
BstXI          EcoRI                                                EcoRI
GCC AGT GTG CTG GAA TTC GGC TT [ PCR Product ] AA GCC GAA TTC TGC AGA TAT
CGG TCA CAC GAC CTT AAG CCG AA                 TT CGG CTT AAG ACG TCT ATA
```

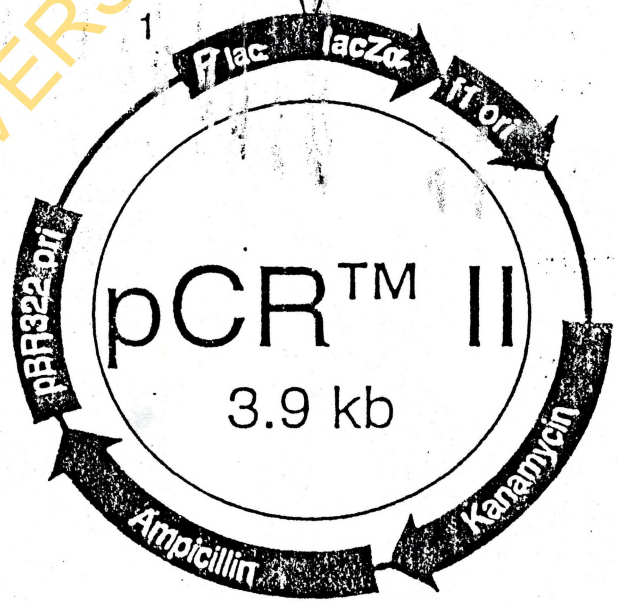
```
                                AvaI
                    XmaIII      PaeR7I
BstXI          NotI             XhoI       NsiI       XbaI       ApaI
CCA TCA CAC TGG CGG CCG CTC GAG CAT GCA TCT AGA GGG CCC AAT TCG CCC TAT
GGT AGT GTG ACC GCC GGC GAG CTC GTA CGT AGA TCT CCC GGG TTA AGC GGG ATA
```

T7 Promoter

```
AGT GAG TCG T T  A C AAT TCA CTG GCC GTC GTT TTA C AA CG T CGT GAC TGG GAA AAC
TCA CTC AGC A A  T G TTA AGT GAC CGG CAG CAA AAT G TT GC A GCA CTG ACC CTT TTG
```

M13 (-20) Forward Primer

M13(-40) Forward Primer



The sequence detailed above represents the pCR™II vector sequence with a PCR product inserted by TA Cloning™.

4.2.10.2. Transformation: 0.5M β-mercaptoethanol (β-ME) was prepared in a chemical hood by diluting 33µl of stock β-ME solution in 1ml sdw and placed on ice. SOC medium was warmed to room temperature while the ligation mixes and frozen competent *E. coli* cells (1 NVaF[1] from Invitrogen) were thawed on ice. Luria-Bertani (LB) 10cm agar plates with antibiotics were placed in a 37°C incubator to remove excess moisture while 2µl of 0.5M β-ME was added to each tube of competent cells and mixed by gently tapping the tubes followed by addition of 4µl of each ligation mixes and gentle mixing of the tube contents. The tubes were incubated on ice for 30 min and heat-shock treated in 42°C water bath for 1 min followed by 2 min incubation on ice. The SOC bottle was briefly disinfected by flaming over a bunsen burner and 450µl of pre-warmed SOC medium were pipeted into each competent cell tube. The tubes were securedly held in a rack and shaken at 37°C at 225 rpm in a gyratory shaker incubator (G24 Environmental Incubator shaker by New Brunswick Sci. Instrument) for 1 hour. The remaining ligation mixes and SOC medium were stored at -20°C while other reagents were stored at -70°C.

While the tubes were being shaken, L-shaped glass spreaders were made from pasture pipets over the bunsen burner flame. LB agar plates (6 for every reactant tube) containing kanamycin or ampicillin at 50µg/ml each were prepared by thinly spreading 25µl of X-Gal (40mg/ml stock solution) on top of the agar with the glass spreaders. The X-Gal was allowed to diffuse into the agar for about 1 hour. After shaking, the tubes containing transformed cells were placed on ice and 100µl of transformed cells were then pipeted onto each agar plate and thinly spread over the agar two or three times to ensure that they dried out completely. The agar plates were covered inverted and incubated at 37°C overnight.

The plates were checked the following day after 24 hours of incubation for blue and white colonies and were then stored at 4°C wrapped in cling film until the colonies were picked.

4.2.10.3. Small scale preparation of plasmid DNA (Mini preps)

Forty eight plastic universal bottles were marked for each virus. Ampicillin at 50µg/ml was added to 500ml LB broth and mixed and 2ml of the broth were pipeted into each of the universal bottles. White colonies of different sizes were picked from the agar plates into the broth with a sterile tooth pick per colony per bottle with each tooth pick left in each universal bottle. The bottle caps were screwed on and the bottles were shaken overnight at 37°C securedly held in an incubator shaker. Similarly, 100-150µl of broth were pipeted into the wells of marked flat bottom 96 well tissue culture plates with a multichannel pipet. A white colony was picked into each well leaving two wells per plate unfilled to serve as controls. The tooth picks were discarded as soon as each colony was dropped into each well. The culture plates were covered and incubated at 37°C overnight as master colonies. They were then preserved with 15% glycerol by adding 35µl of 80% glycerol to each well containing 150µl of culture and 25µl to wells containing 100µl of culture. The plates were covered with plastic adhesive tapes before being covered with their lids to prevent cross contamination.

Mini-preps were then prepared from the overnight cultures. The method used was based on alkaline lyse method described by Birnboim and Doly (1979) in which 500µl of overnight cultures of colonies in plastic universal bottles were pipeted into 1.5ml microfuge tubes (24 tubes per virus) and briefly centrifuged at 14,000 rpm for 10 min in a

microcentrifuge. The supernatant was completely removed and the pellets resuspended in 100µl of solution 1 (50mM glucose, 25mM Tris-HCl pH 8.0, 10mM EDTA pH 8.0). The tubes were left at room temperature for 5 min and 200µl of freshly prepared 1% SDS/0.2M NaOH (solution II) was added, the tubes were vortexed gently and left on ice for 5 min. Half volume (150µl) of 3M potassiun acetate in glacial acetic acid and sdw (solution III) was then added, the tubes were again briefly vortexed to mix the contents and were left on ice for 5 min or longer. They were pelleted for 5 min at 14,000 rpm in a microcentrifuge and the supernatants were transfered into fresh tubes. About 0.6 volume (300µl) of isopropanol was added, vortexed and centrifuged for 1 min at 14,000 rpm to precipitate the DNA. The supernatants were completely removed with fine micro-pipet tips and discarded. The DNA was resuspended in 50µl TE and briefly vortexed. One tenth volume (5µl) of 3M sodium acetate pH 5.0 and three volumes (165µl) of ethanol were added, followed by vortexing to mix and incubation at room temperature for 2 min. The DNA was repelleted by centrifugation for 5 min at 14,000 rpm, the supernatants were removed and the DNA resuspended in 25µl TE and stored at -20°C until required.

The one step rapid method of Chowdhury (1991) was also used for mini-preps. Briefly, TE saturated phenol was prepared by adding equal volume (25ml) of 1M Tris-HCl pH 7.4 to an equal volume (25ml) of water saturated phenol in a 50ml centrifuge tube which was vigorously shaken to mix the contents. A few pinches of 8-hydroxyquinoline were added and allowed to settle down for about 10 min whereby the two solutions separated into two phases. The top Tris-HCl phase was removed and TE (98.8ml sdw, 1ml Tris-HCl pH 7.4, 200µl 0.5M EDTA pH 8.0) was added to 50ml mark. The top TE phase

was removed, care being taken at the interphase. Chloroform and iso-amyl alcohol were added to give 25ml phenol, 24ml chloroform and 1ml iso-amyl alcohol. The tube was centrifuged at 3,000 rpm in a bench top Denley centrifuge for 5 min.

To 500µl of overnight transformed colony cultures from the plastic universal bottles was added 500µl of pheneol:chloroform:iso-amyl alcohol mixture in 1.5ml microfuge tubes. These were mixed by vortexing at maximum speed for 1 min and then centrifuged at 14,000 rpm for 5 min. While being centrifuged, 500µl of isopropanol was pipeted into fresh marked tubes and after centrifugation 450µl from the top phase was carefully pipeted into isopropanol tubes, mixed and centrifuged immediately at 14,000 rpm for 5 min. The supernatant was discarded and 70% ethanol was added to the pellets to wash them. Ethanol was removed and washing was repeated once more, ethanol was again removed completely with drawn-out pipets, the pellets were vacuum dried in a speedvac and resuspended in 100µl TER (Tris-HCl, EDTA and RNAse buffer prepared by dissolving 10mg of RNAse A in 10ml sdw and adding 20µl of this solution to 100µl of Tris-HCl pH 7.4, 20µl of 0.5M EDTA pH 8.0 and 9.8ml sdw to give 10mM Tris-HCl, 1mM EDTA and 2mM RNAse. The mini-prep plasmid DNAs were stored at -20°C until required for digestion.

4.2.10.4 Digestion

The plasmid DNA was cleaved with Eco R 1 restriction enzyme for analysis. All materials used were kept on ice. DNA samples in 20µl volumes per virus were digested in 20µl reactions. A digest master mix was prepared for 20 DNA samples per virus by mixing 40µl of buffer H (90mM Tris-HCl, pH 7,5, 10mM MgCl, 50mM NaCl from

Promega), 40µl acetylated BSA (Promega), 2µl RNAse A at 1µg/µl, 10µl (50 units) of Eco R1 restriction enzyme (Promega) and 280µl sdw. Five µl of DNA was pipeted into marked 0.5ml microfuge tubes and 15µl of digest mix was added, gently mixed and incubated at 37°C for 1 hour. After incubation, 3µl of M13 formamide dye was added to 7µl of digest reaction product which was then checked on 1% agarose with a M_r marker at 75-85 volts for about 1 hour in a minigel apparatus. Ethidium bromide (15µl at 1mg/ml) was added to the TBE running buffer to stain the DNA bands and the gel was stopped when the bromopheneol blue front was just at the bottom of the gel. Bands showing the plasmid vector and DNA inserts were visualised with transmitted UV light and photographed with a polaroid camera. The M_r marker used was Hind III Lamda DNA (Boerhinger) with M_r range of 23.1, 9.4, 6.6, 4.4, 2.3, 2.0, and 0.564 kb.

4.2.11 Genetic Analyses

A technique of multiplex RT/PCR and cycle sequencing was developed for rapid analysis of influenza virus genes (Adeyefa *et al.*, 1994). This technique is based on the conserved terminal sequences of influenza virus segments using oligonucleotides complementary and corresponding to the conserved terminal sequences and thermophilic DNA polymerase following reverse transcription of infected cell or virion RNA. All eight segments of influenza A virus are amplified through PCR and are then subjected to cycle sequencing to generate DNA sequences of the virus genes. The nucleotide sequence of each gene is determined using the dideoxynucleotide chain termination procedure of Sangers *et al.* (1977).

4.2.11.1 Sequencing

Sequencing techniques using both end-labelled primers and direct incorporation of the label (internal labelling) were developed and optimised.

(A) Direct sequencing of cDNA

All materials used were stored on ice through out the experiments. In the end-labelled reaction, 10pmoles of primer was labelled with 10 pmoles of [γ^{32}P]ATP using 5 units of T4 polynucleotide kinase, 1µl of 10X T4 polynucleotide kinase buffer and sdw to 10µl final volume in 0.5ml microfuge tube. The reaction tube was incubated at 37°C for 10-30min, heated at 90°C for 2 min to inactivate the kinase and briefly centrifuged to collect the condensation. Into each of four 0.5ml microfuge tubes marked G, A, T, C was pipeted 1µl of corresponding d/ddNTP mix (see appendix G). In the extension/termination reaction, for each set of four sequencing reactions, 2-3µl of cDNA (PCR products) equivalent to 4-40fmoles was pipeted into a 0.5ml tube. To this was added 4.25µl of 5X sequencing buffer (250mM Tris-HCl pH 9.0, 10mM $MgCl_2$), 1.5µl of labelled primer equivalent to 1.5pmoles and sdw to a final volume of 16µl. The contents were mixed and 5 units (1µl) of Taq DNA polymerase (Promega or Boerhinger) was added, mixed by pipeting up and down or brief spinning. Four µl of this primer/template/enzyme mix were then pipeted into each of the four G, A, T, C tubes containing the d/ddNTP mixes. The tube contents were mixed and 20µl of mineral oil was added to prevent evaporation . The reaction tubes were then placed in a thermal cycler pre-heated at 95°C for 2 min and the cycling programme started. The cycling profile for a primer <24 bases or with a G-C content <50% was 95°C for 30 seconds to denature, 42-52°C for 30 seconds to anneal and

70°C for 1 min to extend, repeated for 30 cycles. For a primer >25 bases the profile was 95°C for 30 sec to denature and 70°C for 30 seconds to anneal and extend, the process being repeated for 30 cycles. The oligonucleotides used for sequencing were 18, 24 or 25mer corresponding to nucleotides 1-18, 1-24, 1-25, 8-30 and 8-31 of influenza virus RNAs. Partial nucleotide sequence analysis of amplified cDNA corresponding to each vRNA segment was carried out using radiolabelled segment specific oligonucleotides in cycle or RNA sequencing. The complete HA gene sequence was determined on the same PCR products used for partial sequencing using labelled oligonucleotides of both plus and minus sense. The additional internal oligonucleotides were based on published sequence data of some H3 equine influenza viruses.

The reaction tubes were held at 4°C after the 30 cycles and the reaction was stopped by adding 3µl of stop solution consisting of 10mM NaOH, 95% formamide, 0.05% bromophenol blue and 0.05% xylene cyanol. The reaction products were sometimes stored at -20°C until required for electrophoresis. The reactions were resolved on 6% acrylamide 8M urea gels run with or without buffer gradient of 0.5X TBE in the top gel tank and 1X TBE in the bottom tank to which was added half volume 3M sodium acetate pH 5.0 after about 1 hour of running the gel when buffer gradient was required in partial nucleotide sequence analysis of the internal genes. Gel were run at a constant wattage of 40 for three and a half hours with buffer gradient. For complete sequence analysis of the HA genes, short and long gels were run for one and a quarter to one and a half hours and 5-6 hours respectively without buffer gradient.

In direct incorporation sequencing reaction, 3 pmoles of primer was added to about 500 fmoles of cDNA followed by 0.5µl of α^{32}P-dATP equivalent to 800 Ci/mmol, 4.25µl of 5X sequencing buffer and sdw to 16µl final volume. Five units (1µl) of Taq DNA polymerase was added and the procedure was completed as described above.

When Vent™ (exo⁻) DNA polymerase was used in end-labelled sequencing reaction, 4-40fmoles of cDNA, 1.5pmoles of labelled primer, 1.5µl of Vent sequencing buffer (100mM KCl, 100mM $\{NH_4\}_2SO_4$, 200mM Tris-HCl pH 8.8, 50mM $MgSO_4$), 1µl 30X Triton X-100 and sdw were added to a final volume of 14µl, 3.2µl of this primer/ template/enzyme mix was added to 3µl of d/ddNTP mix in correspondingly marked G, A, T, C tubes followed by addition of 20µl mineral oil overlay and cycle sequencing. Cycling profile of 95°C for 20 sec to denature, 55°C for 20 sec to anneal and 72°C for 30 sec to extend in 20 cycles was used. The Vent system had the advantage of reducing high background signals and removing artifacts from resolved reactions.

(B) RNA sequencing

Partial nucleotide sequences of vRNA were determined using the dideoxynucleotide chain termination procedure and reverse transcription using end-labelled oligonucleotides. Approximately 1µg of vRNA was used for each reaction with 1µl of the 4 d/ddNTP mixes (400µM dNTPs and 80µM ddNTPs, see appendix G) in a Dynatech 96 well ELISA plate in which 1µl of labelled primer equivalent to 1 pmole was added to 1µl each of the four d/ddNTP mixes. The plate was briefly centrifuged in a microcentrifuge or bench top general purpose centrifuge with a plate adaptor. A reaction mix was prepared for each of

the virus isolate vRNA in a separate 0.5ml microfuge tube containing a total volume of 2.5µl per reaction of 1µg RNA, 5X RT buffer of 10mM KCl, 10mM Tris-HCl pH 8.3, 8mM $MgCl_2$, 5mM DDT final concentration, 10 units of RNAsin (Promega) and 4 units of AMV RTase (Promega). The 2.5µl mix was added to the 2µl d/ddNTP-primer mix, the plate was covered with an adhesive plastic tape and briefly centrifuged before incubation at 42°C for 1 hour. This was followed by addition of 3µl formamide stop solution and incubation at 80°C in a pre-heated oven for 10 min with the plate open. The reactions were then resolved on 6% acrylamide gels with buffer gradient as above.

(C) Plasmid DNA sequencing

About 1µg of DNA obtained from the mini-preps was subjected to cycle sequencing as described above using end-labelled M13 universal forward and reverse primers or non-cycle sequencing using Sequenase^R Version 2.0 (United States Biochemicals). The reaction products were resolved on 6% acrylamide gels as above.

4.2.12 Polyacrylamide gel electrophoresis of nucleic acids

Sequencing gels were cast between glass gel plates of 50cm x 20cm separated by spacers of 0.3mm. The gel solutions were made up from 20% acrylamide, 2% bis-acrylamide, 10M urea and 10X TBE pH 8.3 diluted to 6% for sequencing gels in 1X TBE, 8.3M urea. The gel mixes were polymerised with 0.1% w/v APS and 0.1% TEMED and allowed to set with the appropriate comb in place. Usually 32 or 44 well combs were used. Gels were pre-run for about 30 min. The sequencing reactions at 4° or -20°C were

incubated at 70°C for 2 min before being loaded on to the gels which were run at 40 watts for varying length of time depending on if they were long or short gels and on buffer concentration as described above. Loading order was G, A, T, C for each virus or primer.

The gels were fixed after running in 10% methanol, 10% glacial acetic acid and 80% deionised water for 30 min and vacuum dried on to Whatman 3mm chromatography paper for at least 45-60 min. Radioactivity on wet and dried gels was monitored as above.

4.2.13 Autoradiography

Dried gels were exposed to X-ray films as described in Chapter 2.

4.2.14 Sequence and Phylogenetic analyses

Nucleotide sequences were read from the X-ray films on an illuminator in the order of loading the reaction samples which was G, A, T, C. Partial nucleotide sequence data of the non-glycosylated protein genes were analysed by searching a gene data base by a "best-local-homology" rapid search procedure on a digital array processor to generate 100 or 200 most homologous scores to in order to reveal the relationship of the virus genes to the others in the data base and thus determine their origin.

The complete nucleotide sequences of the HA genes were analysed using the University of Wisconsin Genetic Computer Group (GCG) package of programmes (Deveraux *et al.*, 1984; Higgins *et al.*, 1992). Phylogenetic analysis was done with the distances, neighbour joining and DNAPARS programmes of the PHYLIP package (Felsenstein, 1985, 1989, 1993). The RasMol programme was used to map the amino acid

changes in the viruses' HA molecule to antigenic sites on one monomer.

4.3 RESULTS

4.3.1 Phenol extraction and concentrations of vRNAs

The extracted vRNAs were checked on 1% agarose with transmitted UV light and photographed. The individual RNA segments of vRNA of each of the virus isolate and known influenza virus strains are shown in Plate 4. The vRNA concentrations of the three isolates A/Eq/Ibadan/4/91, A/Eq/Ibadan/6/91 and A/Eq/Ibadan/9/91 were respectively 0.105mg/ml, 0.115mg/ml and 0.055mg/ml equivalent to 0.105µg/µl, 0.115µg/µl and 0.055µg/µl.

4.3.2 Concentrations of infected cell RNAs

The concentrations of i.c. RNAs calculated from their absorbance values of 0.370, 0.327 and 0.322 and the formula given in 4.2.9 above were respectively 1.85mg/ml. 1.64mg/ml and 1.61mg/ml equivalent to 1.85µg/µl, 1.64µg/µl and 1.61µg/µl. The ratios of A_{260}:A_{280} for the viruses were 1.73, 1.88 and 1.78 while the ratios of A_{260}:A_{230} were 2.5, 2.3 and 2.12, respectively as calculated from their A_{280} of 0.213, 0.174 and 0.181 and their A_{230} of 0.147, 0.143 and 0.152, respectively.

The concentrations of both vRNA and i.c RNA of the virus isolates were within the average values obtained for influenza viruses. The ratios of absorbance values at 260nm to values at 280nm indicate good quality RNAs while the ratios at 260nm and 230nm imply that there was no contamination of the extracted RNAs (Promega, 1991).

The working stocks of 1µg/µl of i.c. RNAs were calculated as shown in Table 9.

Plate 4. Virion RNA of Ibadan equine and other influenza A viruses.

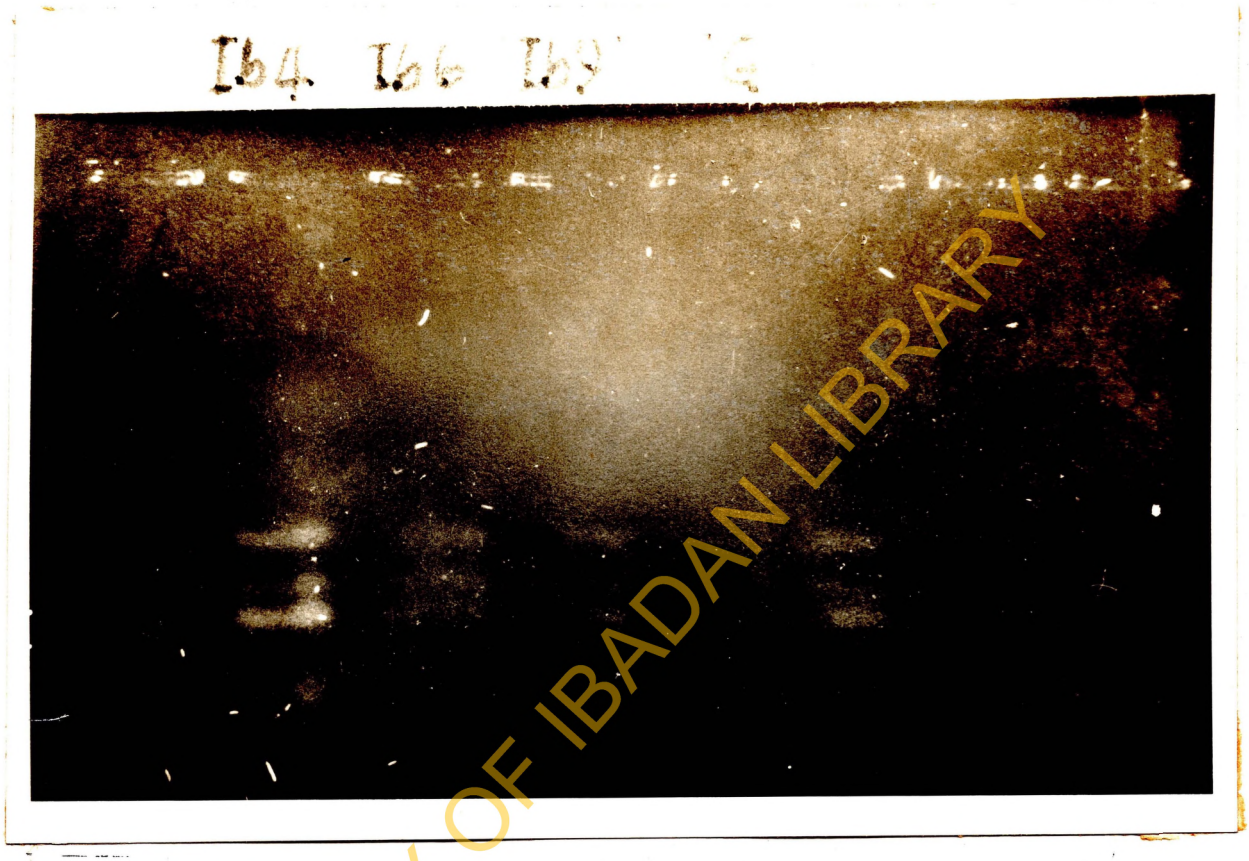


Table 9. Concentration of working stock of infected cell RNAs

Virus	A_{260} Values	Inf. cell RNA Concentration	Volume of Inf. cell RNA in µl	Volume of MQ in µ	Total Volume in µl	Final Working Conc.
A/Eq/Ib/4/91	0.370	1.85µg/µl	$\frac{50}{1.85} \times 1$ = 27.63	50-27.03 = 22.97	50	1µg/µl
A/Eq/Ib/6/91	0.327	1.64µg/µl	$\frac{50}{1.64} \times 1$ = 30.5	50-30.5 = 19.5	50	1µg/µl
A/Eq/Ib/9/91	0.322	1.61µg/µl	$\frac{50}{1.61} \times 1$ = 31.95	50-31.05 = 18.95	50	1µg/µl

4.3.3 Synthesis, purification, deprotection and concentration of oligonucleotide primers

A total of forty oligonucleotide primers were used in this study, eleven (ET primers) of which were kindly provided by Dr. R.G. Webster, St. Jude's Children Research Hospital, Memphis, Tennessee, U.S.A, one, oligo dT was purchased from Sigma Corporation, while the remaining were synthesised at the Pirbright and Compton Laboratories of the Institute for Animal Health, U.K. Primer lengths, composition and sequences are shown in the appendix F while the concentrations of the primers used in this study are shown in Table 10. All the primers used for sequencing were used at a concentration of 10pmoles in the labelling reactions and at 1.5-2pmoles in the extension/termination reactions.

4.3.4 Reverse transcription

The reverse transcripts of individual RNA segments of the three virus isolates and other known equine and avian influenza A viruses are shown in Plate 5. Eight RNA segments were seen as transcripts of genomic RNAs. The estimated average M_r of the RNAs of the three virus isolates in kilobase (Kb) were: Seg 1 and 2 (PB2, PB1) 2.34, Seg 3 (PA), 2.23, Seg 4 (HA), 1.76, Seg 5 (NP), 1.56, Seg 6 (NA), 1.45, Seg 7 (M), 1.03 and Seg 8 (NS), 0.89 Kb. The average total M_r for the three virus isolates was 13.6 Kb which was within the normal range for influenza A viruses (Akkina, 1990). Taken together, the 8 RNA transcripts and their estimated M_r confirm that the three isolates were influenza A viruses of equine origin. No defective interfering/subgenomic RNAs were observed in the transcript X-ray films.

Plate 5. Reverse transcription of Ibadan equine influenza virus RNA segments showing the individual 8 RNA segments encoding virus polypeptides.

Segment	1	encodes basic protein	PB2	a=A/Eq/Prague/56 (H7N7)
"	2	encodes basic protein	PB1	b=A/Eq/Miami/63 (N3N8)
"	3	encodes acidic protein	PA	c=A/FPV/Rostock/34 (H7N7)
"	4	encodes Haemagglutinin	HA	d=A/Eq/Ibadan/4/91 (H3N8)
"	5	encodes nucleoprotein	NP	e=A/Eq/Ibadan/6/91 (H3N8)
"	6	encodes neuraminidase	NA	f=A/Eq/Ibadan/9/91 (H3N8)
"	7	encodes matrix protein	M	
"	8	encodes non-structural protein	NS	molecular weights in kilobase (Kb) are shown on the right

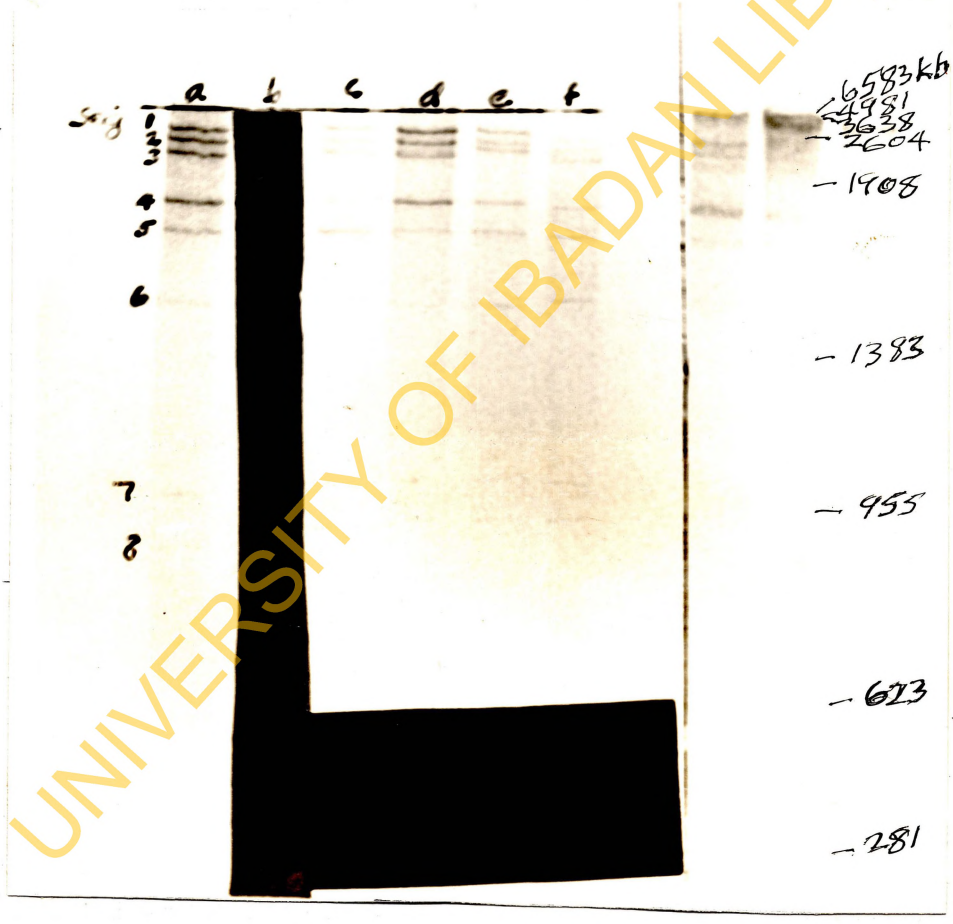


Table 10. Oligonucleotide primers and their concentrations

Primers	Concentrations
First strand primer: 3'-1-12	1.4pmoles/µl
Second strandprimer: 5'-1-13	2.98pmoles/µl
Oligo dT (Sigma)	4.2pmles/µl
Segment 1: 3'	40µM
Segment 2: 3'	40µl
Segment 3: 8-27	243pmoles/µl
Segment 5: NP1-24	406pmoles/µl
Segment 6: N8-26	104.2pmoles/µl
Segment 7: 1-25	64pmoles/µl
Segment 8: V8 8-31	69.44pmoles/µl
H3Eq/HA 1: 8-27	10µM
H3Eq/HA 101-124	280pmoles/µl
H3Eq/HA 2: 151-170	10µM
H3Eq/HA 3: 315-333	1.58nmoles/µl
H3Eq/HA 4: 481-498	1.2nmoles/µl
H3Eq/HA 5: 661-680	1.3nmoles/µl
H3Eq/HA 6: 841-859	1.2nmoles/µl
H3Eq/HA 7	408.77pmoles/µl
H3Eq/HA 8	426pmoles/µl
H3Eq/HA 9 Reverse	325.4pmoles/µl
H3Eq/HA 200 Reverse	457.8pmoles/µl
H3Eq/HA 250 Reverse	377.8pmoles/µl
H3eq/HA 650	340pmoles/µl
H3Eq/HA 701	429.6pmoles/µl
H3Eq/HA 950 Reverse	389pmoles/µl
H3Eq/HA 1000 Reverse	452pmoles/µl
H3Eq/HA1740reverse	352.6pmoles/µl
ET 9	2.5µg
ET 268	2.5µg
ET 444	2.5µg
ET 592	2.5µg
ET 714	2.5µg
ET 879	2.5µg
ET 1032	2.5µg
ET 1182	2.5µg
ET 1277	2.5µg
ET 1368	2.5µg
ET 1526	2.5µg
pUC/M13 Universal forward	1µg
pUC/M13 Universal Reverse	1µg

4.3.5 Generation of cDNA through PCR amplification and purification of PCR products

The products of PCR reactions are shown in Plate 6. All of the 8 RNA segments had been amplified, although it is not necessary for all the segments to be visible in the PCR products visualised with transmitted light or in the photograph as seen in Plate 6 for the subsequent sequencing to be successful (Adeyefa *et al.*, 1994). The PCR products are very similar to those of other influenza A viruses of human, avian and equine origin as earlier shown by Adeyefa *et al* (1994). Attempts to detect virus RNAs/cDNA directly from the clinical nasal swabs gave variable results which were sometimes not quite satisfactory (photograph not shown).

4.3.6. Cloning of virus cDNA in plasmid vectors

The full length copy of each RNA segment of the three virus isolates was successfully cloned into the PCR™ TA Cloning vector. Plate 7 shows the cloned genes (inserts) and the cloning vector. Each of the 8 virus genes was thus cloned.

4.3.7 Sequense analysis and the origin of the genes of virus non-glycosylated polypeptides

Plate 8 shows an example of nucleotide sequence ladder of Ibadan virus genes. Partial nucleotide sequences (Fig. 17) were obtained from cDNA, and plasmid DNA corresponding to segments 1, 2, 3, 5, 7 and 8 by use of the rapid method for analysing influenza virus genes based on multiplex RT/PCR and cycle sequencing (Adeyefa *et*

Plate 6. PCR products of infected cell RNA of Ibadan equine and other influenza A viruses

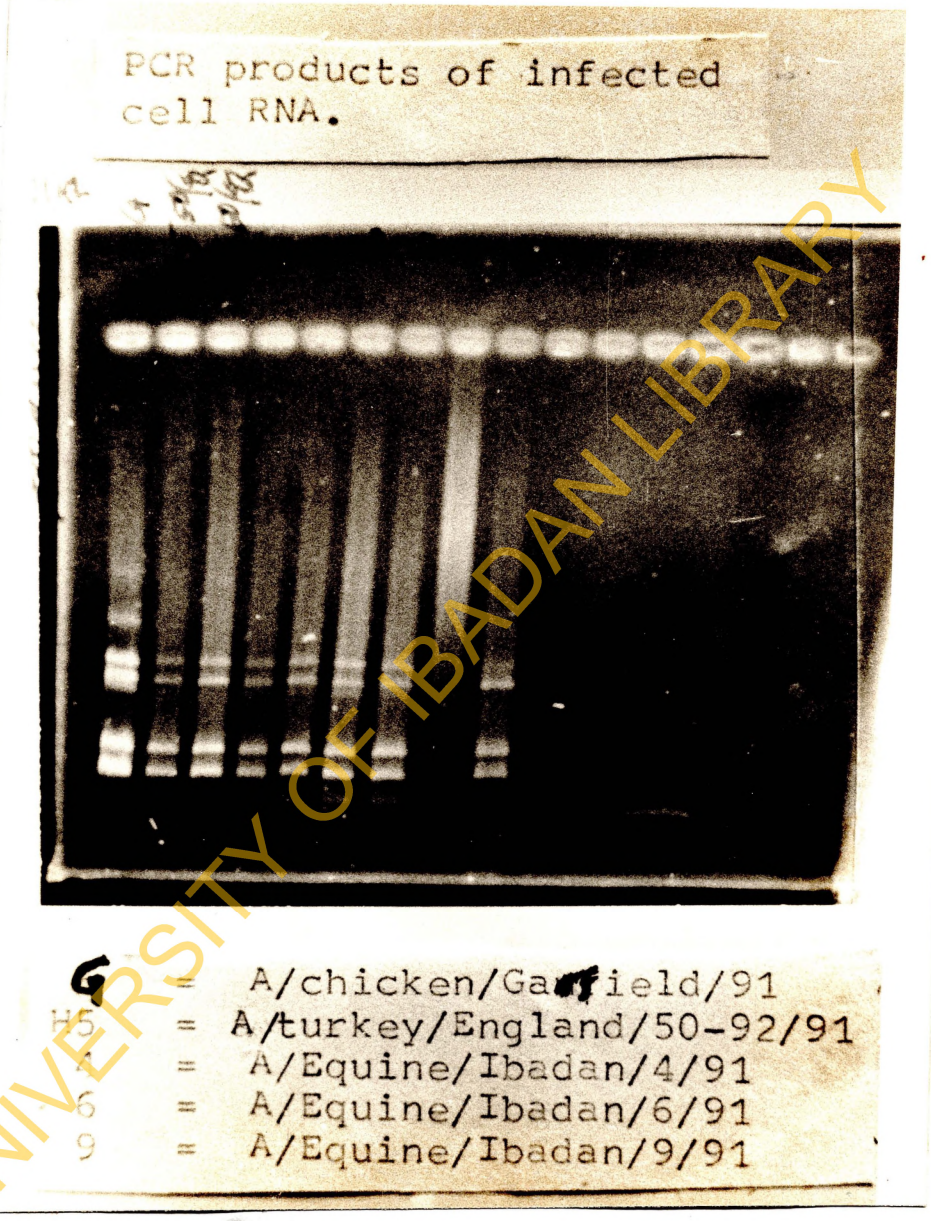


Plate 7. Cloned genes of Ibadan equine influenza virus isolates and the cloning vector. Individual genes and the 3.9Kb TA cloning vector are highlighted.

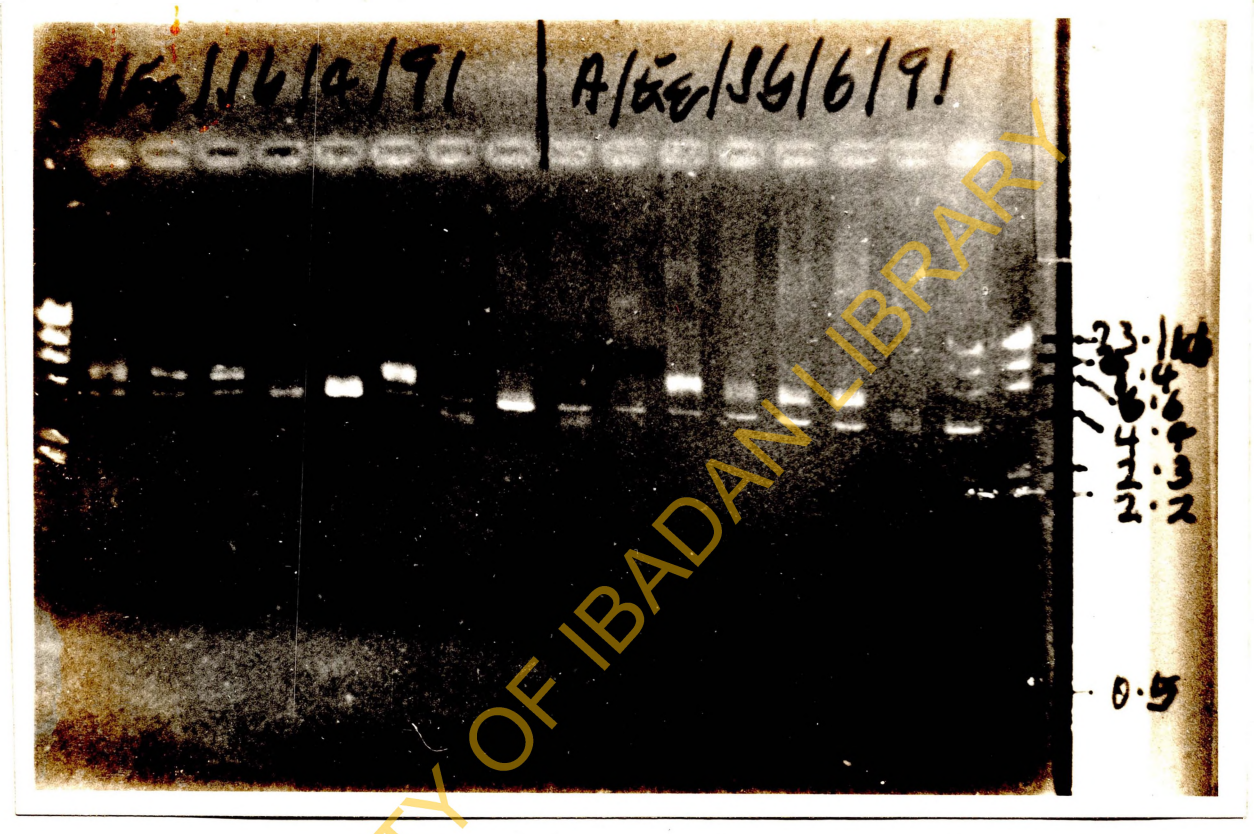


Plate 8. Nucleotide sequence ladder of Segment 7 (M) gene of A/Eq/Ibadan/4/91 influenza virus from nucleotides 70-89 with primer Seg. 7:1-25. The order of loading is G, A, T, C and sequences are read from the bottom to the top of the gel as indicated on the right.

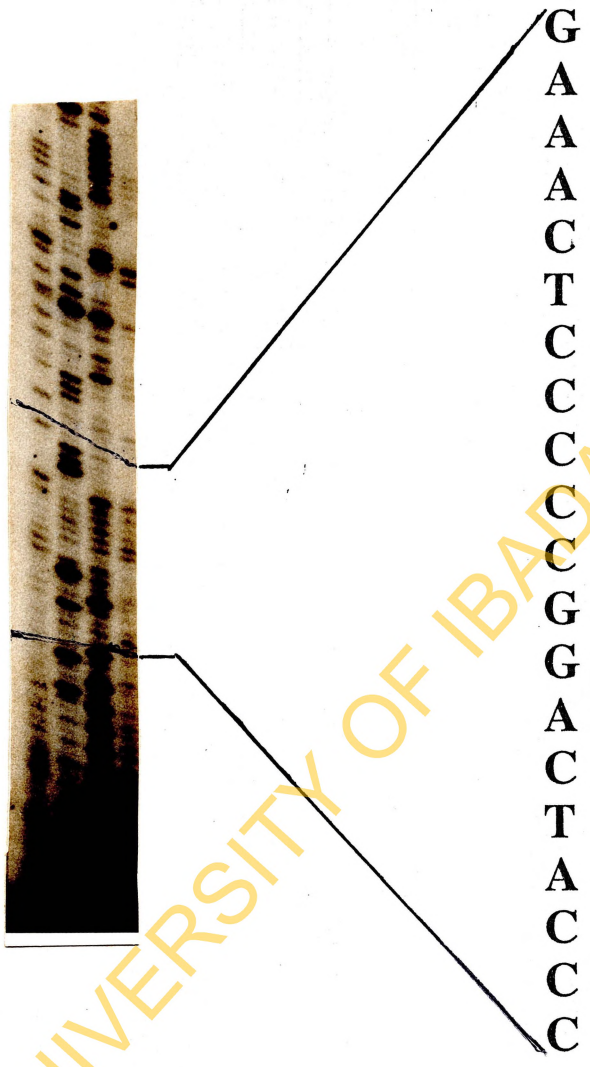


Fig. 16. Partial nucleotide sequences of Segments 1, 2, 3, 5, 7 and 8 of Ibadan equine influenza viruses.
(..... indicate sequences not available on the gels, boxed sequences in Seg. 7 refer to Plate 8)

SEGMENT 1 (PB2)

	1	10	20	30	40	50
Prague 56	a g c a a a a g c a	g g t c a a a t a t	a t t c a a t a t g	g a g a g a a t a g	a a g a a a c t a a g
London 141673	a g c a a a a g c a	g g t c a a a t a t	a t t c a a t a t g	g a g a g a a t a a	a a g a a a c t g a g
Ken 286	a g c a a a a g c a	g g t c a a a t a t	a t t c a a t a t g	g a g a g a a t a a	a a g a a a c t g a g
Ibadan 491	. . . . .	. . . . .	. . . . .	g a g . a g a g t a a	a a g a a a c t g a g
Ibadan 691	. . . . .	. . . . .	. . . . .	. . . . . a a t a a	a a g a a a c t g a g
Ibadan 991	. . . . .	. . . . .	. . . . .	. . . . . a a	a a g a a a c t g a g

	51	60	70	80	90	100
Prague 56	a g a t t t g a t g	t c a c a a t c c c	g c a c t c g c g a	g a t a c t a a c a	a c a a c c a c c g
London 141673	a g a t c t a a t g	t c a c a g t c c c	g c a c c c g c g a	g a t a c t c a c a	a a a a c c a c t g
Ken 286	a g a t c t a a t g	t c a c a g t c c c	g c a c c c g c g a	g a t a c t a a c a	a a a a c t a c t g
Ibadan 491	a g a t c t a a t g	t c a c a g t c c c	g c a c c c g c g a	g a t a c t a a c a	a a a a c c a c t g
Ibadan 691	a g a t c t a a t g	t c a c a g t c c c	g c a c c c g c g a	g a t a c t a a c a	a a a a c t a c t g
Ibadan 991	a g a t c t a a t g	t c a c a g t c c c	g c a c c c g c g a	g a t a c t a a c a	a a a a c a c t g

	101	110	120	130	140	150
Prague 56	t g g a c c a c a t	g g c c a t a a t t	a a a a g g t a c a	c a t c a g g a a g	a c a a g a a a a g
London 141673	t g g a c c a t a t	g g c c a t a a t c	a a g a a a t a c a	c a t c a g g a a g	a c a a g a g a a g
Ken 286	t g g a c c a t a t	g g c c a t a a t c	a a g a a a t a c a	c a t c a g g a a g	a c a a g a g a a g
Ibadan 491	t g g a c c a t a t	g g c c a t a a t c	a a g a a a t a c a	c a t c a g g a a g	a c a a g a g a a g
Ibadan 691	t g g a c c a t a t	g g c c a t a a t c	a a g a a a t a c a	c a t c a g g g a g	a c a a g a g a a g
Ibadan 991	t g g a c c a c a t	g g c c a t a a t t	a a g a a a t a c a	c a t c a g g a a g	a c a a g a g a a g

	151					200
Prague 56	a a c c c a g c a t	t a a g a a t g a a	a t g g a t g a t g	g c a a t g a a a t	a c c c a a t c a c
London 141673	a a c c c c g c a c	t t a g g a t g a a	g t g g a t g a t g	g c a a t g a a a t	a t c c a a t t a c
Ken 286	a a c c c c g c a c	t t a g g a t g a a	g t g g a t g a t g	g c a a t g a a a t	a c c c a a t t a c
Ibadan 491	a a c c c c g c a c	t t a g g a t g a a	g t g g a t g a t g	g c a a t g a a a t	a c c c a a t t a c
Ibadan 691	a a c c c c g c a c	t t a g g a t g a a	g t g g a t g a t g	g c a a t g a a a t	a c c a a a t t a c
Ibadan 991	a a c c c a g t a c	t t a g g a c g a a	a t g g a t g a t g	g c a a t g a a a t	a c c c a a t c a c

SEGMENT 2 (PB1)

	1	10	20	30	40	50
London 141673	a g c a a a a g c a	g g c a a a c t a t	t t g a a t g g a t	g t c a a c c c g a	c t c t a c t c t t
Tenn 86	a g c a a a a g c a	g g c a a a c t a t	t t g a a t g g a t	g t c a a t c c g a	c t c t a c t c t t
Ibadan 491	. . . . .	. . . . . t a t	t t g a a t g g a t	g t c a a t c c g a	c t c t a c t c t t
Ibadan 691	. . . . .	. . . . .	. . . . .	g t c a a c c c g a	c c c t a c t c t t
Ibadan 991	. . . . .	. . . . .	. . . . a t g g t t	g t c c a t c c g a	c c c t a a c c t t

	51	60	70	80	90	100
London 141673	c t t a a a a g t g	c c a g c g c a a a	a t g c t a t a a g	c a c a a c a t t c	c c t t a t a c t g
Tenn 586	c t t a a a a g g t g	c c a g c g c a a a	a t g c t a t a a g	c a c a a c a t t c	c c t t a t a c t g
Ibadan 491	c t t a a a a g g t g	c c a g c g c a a a	a t g c t a t a a g	c a c a a c a t t c	c c t t a t a c t g
Ibadan 691	c t t a a a a g t t g	c c a g c g c a a a	a t g c t a t a a g	c a c a a c a t t c	c c t t a t a c t g
Ibadan 991	c t t a a a a g t g	c c a g c a c a a a	a t g c t a t a a g	c a c a a c a t t c	c c t t a c a c t g

SEGMENT 2 (PB1) contd.

	110	120	130	140	150
London 141673	gagatcctcc	ctacagtcat	ggaacaggaa	caggatacac	catggatact
Tenn 586	gagatcctcc	ctacagtcat	ggaacaggga	caggatacac	catggatact
Ibadan 491	gagatcctcc	ctacagtcat	ggaacaggga	caggatacac	catggatact
Ibadan 691	gagatcctcc	ctaaagtcat	ggaacaggga	caggataaac	catggatact
Ibadan 991	gagatcctcc	ctaaagtcat	ggaacaggga	caggataaac	catggatact

	160	170	180	190	200
London 141673	gtcaacagaa	cacaccaata	ctcaggaaaag	gggaaatgga	caacaaacat
Tenn 586	gtcaacagaa	cacaccaata	ctcaggaaaag	gggaaatgga	caacaaacat
Ibadan 491	gtcaacagaa	cacaccaata	ctcaggaaaag	gggacatgga	caacaaacat
Ibadan 691	gtcaacagaa	cacaccaata	ctcagaaagg	gggacatgga	caacaaacat
Ibadan 991	gtcaacagaa	cacactaata	ctcaggaagg	gggaaatgga	caacaaccat

SEGMENT 3 (PA)

	10	20	30	40	50
London 141673	agcaaaagca	ggtactgatc	caaaatggaa	gactttgtgc	gacaatgctt
Tenn 586	agcaaaagca	ggtactgatc	caaaatggaa	gactttgtgc	gacaatgctt
Ibadan 491	.....	...tactgatc	caaaatggaa	gactttgtgc	gacaatgctt
Ibadan 691	.....	.....	caaaatggaa	gactttgtgc	gacaatgctt
Ibadan 991	.....ca	ggtactgatc	caaaatggaa	gactttgtgc	gacaatgctt

	60	70	80	90	100
London 141673	caatccaatg	atcgtcgagc	ttgcggaaaa	ggcgatgaaa	gaatatggag
Tenn 586	caatccaatg	atcgtcgagc	ttgcggaaaa	ggcaatgaaa	gaatatggag
Ibadan 491	caatccaatg	atcgtcgagc	ttgcggaaaa	ggcaatgaaa	gaatatggag
Ibadan 691	caatccaatg	atcgtcgagc	ttgcggaaaa	ggcaatgaaa	gaatatggag
Ibadan 991	caatccaatg	atcgtcgagc	ttgcggaaaa	ggcgatgaaa	gaatatggag

	110	120	130	140	150
London 141673	aggacccgaa	aattgaaaca	aacaaatttg	cagcaatatg	cactcacttg
Tenn 586	aggatccgaa	aattgaaaca	aacaaatttg	cagcaatatg	cactcacttg
Ibadan 491	aggacccgaa	aattgaaaca	aacaaatttg	cagcaatatg	cactcacttg
Ibadan 691	aggacccgaa	aattgaaaca	aacaaatttg	cagcaatatg	cactcacttg
Ibadan 991	aagacccgaa	aattgaaaca	aacaaatttg	cagcaatatg	cactcacttg

	160	170	180	190	200
London 141673	gaagtctgct	tcatgtactc	ggattttccac	tttattaatg	aactgggcga
Tenn 586	gaagtctgct	tcatgtactc	ggattttccac	tttattaatg	aactgggtga
Ibadan 491	gaagtctgct	tcatgtactc	ggattttccac	tttattaatg	aactgggtga
Ibadan 691	gaagtctgct	tcatgtactc	ggattttccac	tttattaatg	aactgggtga
Ibadan 991	gaagtctgct	tcatgtactc	ggattttccat	tttattaatg	aactgggtga

SEGMENT 5 (NP)

```
                    10         20         30         40         50
London1461673  agcaaaagca gggtagataa tcactcactg agtgacatca aactcatggc
Tenn 586       agcaaaagca gggtagataa tcactcactg agtgacatca aactcatggc
Ken 286        agcaaaagca gggtagataa tcactcactg agtgacatca aagtcatggc
Miami 63       agcaaaagca gggtagataa tcactcactg agtgacatca aactcatggc
Gull MD577     agcaaaagca gggtagataa tcactcactg agtgacatcc acaacatggc
Ibadan491      agcaaaagca gggtagataa tcactcaxtg agtgacatca aactcatggc
Ibadan691      ...........gggtagatta tcactcactg agtgacatca aagtcxxggc
Ibadan991      ..........  .....xtcactg agtgacatca aactcatgac

                    60         70         80         90        100
London1461673  gtctcaaggc accaaacgat cttatgagca gatgggaact gggtgggaac
Tenn 586       gtctaaaggc accaaacgat cttatgagca gatgggaact gggtgggaac
Ken 286        gtctcaaggc accaaacgat cttatgagca gatgggaact gggtgggaac
Miami 63       gtctcaaggc accaaacgat cttatgagca gatgggaact gggtgggaac
Gull MD577     gtctcaaggc accaaacgat cttatgagca gatgggaact gggcgggaac
Ibadan491      gtctaaaggc accaaacgat cttatgagca gatgggaact gggtgggaac
Ibadan691      gtctcaaggc accaaacgat cttatgagca gatgggaact gggtgggaac
Ibadan991      gtctcaaggc accaaacgat cttatgagca gatgggaact gggtgggaac

                   110        120        130        140        150
London1461673  gccagaatgc aactgaaatc agagcatctg ttgggagggat gggtgggagga
Tenn 586       gccagaatgc aactgaaatc agagcatctg tcgggagggat gggtgggagga
Ken 286        gccagaatgc aactgaaatc agagcatctg tcgggagggat gggtgggagga
Miami 63       gccagaatgc tactgagatc agagcatctg tcgggagggat gggtgggagga
Gull MD577     gccagaatgc tactgagatc agagcattcg ttgggagaat gggtcgggtgga
Ibadan491      gccagaatgc aactgagatc agagcatctg tcgggagggat gggtgggagga
Ibadan691      gccagaatgc aactgagatc agagcatctg tcgggagggat gggtgggagga
Ibadan991      gccagaatgc tactgagatc agagcatctg tcgggagggat gggtgggagga

                   160        170        180        190        200
London1461673  atcggccggt ttcatgttca gatgtgtact gagctcaaac tcaacgacca
Tenn 586       atcggccggt tctatgttca gatgtgtact gaggcttaac tcaacgacca
Ken 286        atcggccggt tctatgttca gatgtgtact gaggcttaac tcaacgacca
Miami 63       atcggccggt tctatgttca gatgtgtact gaggcttaac tcaacgacca
Gull MD577     attggcaggt tctacattca gatgtgcact gaggctcaac tcaggcacta
Ibadan491      atcggccggt tctatgttca gatgtgtact gaggcttaac tcaacgacca
Ibadan691      atcggccggt tctatgttca gatgtgtact gaggcttaac tcaacgacca
Ibadan991      atcggcaggt tctatgttca gatgtgtact gaggcttaac tcaacgacca

                   210        220        230        240        250
London1461673  tgaaggggcgg ctgattcaga acagcataac aatagaggagg atggtgcttt
Tenn 586       tgaaggggcgg ctgattcaga acagcataac aatagaaaggg atggtacttt
Ken 286        tgaaggggcgg ctgattcaga acagcataac aatagaaaggg atggtacttt
Miami 63       tgaaggggcgg ctgattcaga acagcataac aatagaaaggg atggtacttt
Gull MD577     tgaaggggagg ctgattcaga acagcataac aatagagagga atggttcttt
Ibadan491      tgaaggggagg ctgatccaga acagcataac aatagaaaggg atggtacttt
Ibadan691      tgaaggggcgg ctgattcaga acagcataac aatagaaaggg atggtacttt
Ibadan991      tgaaggggcgg ctgattcaga acagcataac aatagaaaggg atggtg...
```

SEGMENT 7 (M)

```
                    10         20         30         40         50
Tenn 586    agcaaaaagca ggtagatatt taaagatgag tcttctgacc gagggtcgaaa
Ken 286     agcaaaaagca ggtagatatt taagagatgag tcttctaacc gagggtcgaaa
Ibadan491   ...........  .......... ...agatgag tcttctgacc gagggtcgaaa
Ibadan691   ...........  ......agat attaagatgag tcttctgacc gagggtcgaaa
Ibadan991                                      cttctgacc gagggtcgaac

                    60         70         80         90        100
Tenn 586    cgtacgttcc ctctatcgta ccatcaggcc ccctcaaagc cgagatcgcg
Ken 286     cgtacgttcc ctctattgta ccatcaggcc ccctcaaagc cgagatcgcg
Ibadan491   cgtacgttcc ctctatcgtc ccatcaggcc ccctcaaagc cgagatcgcg
Ibadan691   cgtacgttcc ctctatcgta ccatcaggcc ccctcaaagc cgagatcgcg
Ibadan991   cgtacgttcc ctctatcgta ccatcaggcc ccctcaaagc cgagatcgcg

                   110        120        130        140        150
Tenn 586    cagagacttg aagatgtctt tgcagggaag aacaccgatc ttgaggcact
Ken 286     cagagacttg aagatgtctt tgcagggaag aacaccgatc ttgaggcact
Ibadan491   cagagacttg aagatgtctt tgcagggaag aacaccgatc ttgaggcact
Ibadan691   cagagacttg aagatgtctt tgcagggaag aacacgaatc ttgaggcact
Ibadan991   cagagacttg aagatgtctt tgcagggaag aacaccgatc ttgaggcact

                   160        170        180        190        200
Tenn 586    catggaatgg ctaaagacga gaccaatcct gtcacctctg actaaaggga
Ken 286     catggaatgg ctaaagacaa gaccaatcct gtcacctctg actaaaggga
Ibadan491   catggaatgg ctaaagacaa gaacaatcct gtcacctctg actaaaggga
Ibadan691   catggaatgg ctaaagacaa gaacaatcct gtcacctctg actaaag...
Ibadan991   catggaatgg ctaagacaa gaacaatcct gtcacctctg ..........
```

SEGMENT 8 (NS)

```
                    10         20         30         40         50
Miami 63    agcaaaaagca gggtgacaaa xxxx..atgg attccaacac tgtgtcaagc
Nmlt 76     agcaaaaagca gggtgacaaa aacataatgg attccaacac tgtgtcaagc
Ibadan491   ..........  .gtgacaaa aacataatgg attccaacac tgtgtcaagc
Ibadan691   ..........  .......aa aacataattg axxccaacac tgtgtcaagc
Ibadan991   ...aaaagca gggtgacaaa aacataattg attccaacac tgtgtcaagc

                    60         70         80         90        100
Miami 63    ttcaggtag actggttctct ttgg catgtc cgcaaacgat ttgcagacca
Nmlt 76     ttcaggtag actggttctct ttgg catgtc cgcaaacgat ttgcagacca
Ibadan491   ttcaggtag actggttctct ttgg catgtc cgcaaacgat ttgcagacca
Ibadan691   ttccggtag actgattctct ttgg catgtc cgcaaacgat ttgcagacca
Ibadan991   ttccggtag actgattctct ttgg catgtc cgcaaacgat ttgcagacca

                   110        120        130        140        150
Miami 63    agaactgggt gatgccccat tccttgaccg acttcgccga gaccagaagt
Nmlt 76     agaactgggt gatgccccat tccttgaccg gcttcgccga gaccagaagt
Ibadan491   agaactgggt gatgccccat tccttgaccg gcttcgccga gaccagaagt
Ibadan691   agaactgggt gatgccccat tccttgaccg gcttcgccga gaccaa....
Ibadan991   agaactgggt gatgcccaat tccttgaccg gcttcgccga gaccagaagt
```

al., 1994). Partial nucleotide sequences were also obtained from RNA sequencing of these segments and were the same as for cDNA and plasmid DNA sequences. The partial cDNA sequences of about 200-250 nucleotides were then compared with the virus DNA sequences in the European Molecular Biology Library (EMBL) sequence database. The results of the database homology scores are summerised in Tables 11-13. The results show that for segments 1, 2, 5 and 7 the closest related sequence was from equine H3 viruses isolated during 1986 in USA (A/Eq/Tennessee/5/1986) for segments 2 and 7 and A/Eq/Kentucky/2/86 for segment 1 while segment 5 was equally related to each of the 1986 viruses. The nucleotide sequence for segment 3 was most closely related to an H7N7 equine-1 virus isolated in 1973 in UK (A/Eq/London/1416/73) but it is known that there had been genetic shift in the internal genes of equine H7N7 viruses isolated after 1972 in which those of the RNP complex were usurped by those of the H3N8 subtype (Bean, 1984; Okazaki *et al.*, 1989; Gorman *et al.*, 1990, 1991, Adeyefa *et al.*, 1994). RNA segment 8 sequence was most closely related to an equine H3N8 virus isolated in UK in 1976 (A/Eq/Newmarket/76). Overall, these findings on the posible origin of the internal genes of these Ibadan viruses group them with viruses previously present in the Western Hemisphere but they do not imply identity with previous strains for which nucleotide sequences are available in the database.

4.3.8 Complete nucleotide sequence analysis of the haemagglutinin genes

The complete nucleotide sequences of the HA genes of the three Ibadan viruses were determined following the amplification of cDNA by the multiplex

Table 11. Homology search results from comparison with Eq/Ibadan/4/91.

	PROBE SIZE	MATCH No.	VIRUS MATCHED		MATCH	MIS-MATCH	%
SEGMENT 1	100 BP	1	Eq/KY/2/86	H3N8	100	0	100
		2	Eq/Lond/1416/73	H7N7	98	2	98
		3	Gull/MD/704/77	H13N6	94	6	94
SEGMENT 2	111 BP	1	Eq/Tn/5/86	H3N8	110	1	98
		2	Eq/Lond/1416/73	H7N7	109	2	97
		3	SW/HK/126/82	H3N2	101	10	89
SEGMENT 3	129 BP	1	Eq/Lond/1416/73	H7N7	128	1	99
		2	Eq/Tn/5/86	H3N8	125	4	97
		3	FPV/Rostock/34	H7N7	119	10	92
SEGMENT 5	147 BP	1	Eq/KY/2/86	H3N8	141	4	96
		2	Eq/TN/5/86	H3N8	141	4	96
		3	Eq/Lond/1416/73	H7N7	139	6	95
		4	Eq/Miami/1/63	H3N8	133	8	90
		5	Gull/MD/704/77	H13 N6	127	17	86
		6	Duck/PENN/1/69	H6N1	125	16	85
SEGMENT 8	146 BP	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
		4	Duck/Eng/56	H11N6	140	6	96

Table 12. Homology search results from comparison with A/Eq/Ibadan/6/91.

	PROBE SEQUENCE	MATCH No.	VIRUS MATCHED		MATCH	MIS-MATCH	%
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/NY/78	H3N2	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	48-194	1	Eq/Lond/1416/73	H7N7	129	1	98.5
		2	Eq/Tenn/5/86	H3N8	126	4	96.2
		3	WSN/33/34	H1N1	119	8	93.2
SEGMENT 5	48-195	1	Eq/Tenn/5/86	H3N8	143	5	96.6
		2	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/6570/78	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	Mynahh/Thai/76	H3N1	140	6	96
		4	Duck/Eng/56	H11N6	140	6	96

Table 13. Homology search results from comparison with A/Eq/Ibadan/9/91.

	PROBE SIZE	MATCH No.	VIRUS MATCHED		MATCH	MIS-MATCH	%
SEGMENT 1	100	1	A/Eq/KY/2/86	H3N8	99	1	99
		2	A/Eq/Lond/1416/73	H7N7	96	4	96
		3	Gull/MD/704/77	H13N6	94	6	94
SEGMENT 2	120	1	Eq/Tenn/5/86	H3N8	119	1	99.1
		2	Eq/Lond/1416/73	H7N7	117	3	97.5
		3	Swine/HK/126/82	H3N2	111	9	92.5
SEGMENT 3	130	1	Eq/Lond/1416/73	H7N7	129	1	99.2
		2	Eq/Tenn/5/86	H3N8	127	3	97.6
		3	SN/Ger/2/81	H1N1	126	5	96
SEGMENT 5	147	1	Eq/Tenn/5/86	H3N8	142	5	96.5
		2	Eq/KY/2/86	H3N8	141	6	96
		3	Eq/Lond/1416/73	H7N7	141	6	96
		4	Eq/Miami/1/63	H3N8	143	4	97.2
		5	Gull/MD/5/77	H11N9	128	19	87
SEGMENT 7	145	1	Eq/Tenn/5/86	H3N8	143	2	98
		2	Eq/Ky/2/86	H3N8	143	2	98
		3	Gull/MD/704/77	H13N6	128	17	86
SEGMENT 8	150	1	Eq/NMKT/76	H3N8	147	3	98
		2	Eq/Miami/1/63	H3N8	149	1	99
		3	Duck/Eng/56	H11N6	140	10	93.3

Fig. 17 A,B and C: Complete nucleotide sequence of the HA gene and amino acid sequense of HA glycoprotein of Ibadan equine influenza viruses **(see legend on page 180h)**

(A) Nucleotide and amino acid sequences of A/Eq/Ibadan/4/91 (Ib4) and A/Eq/Ibadan/6/91 (Ib6) with sequence differences indicated (nucleotides in orange, amino acids in green).

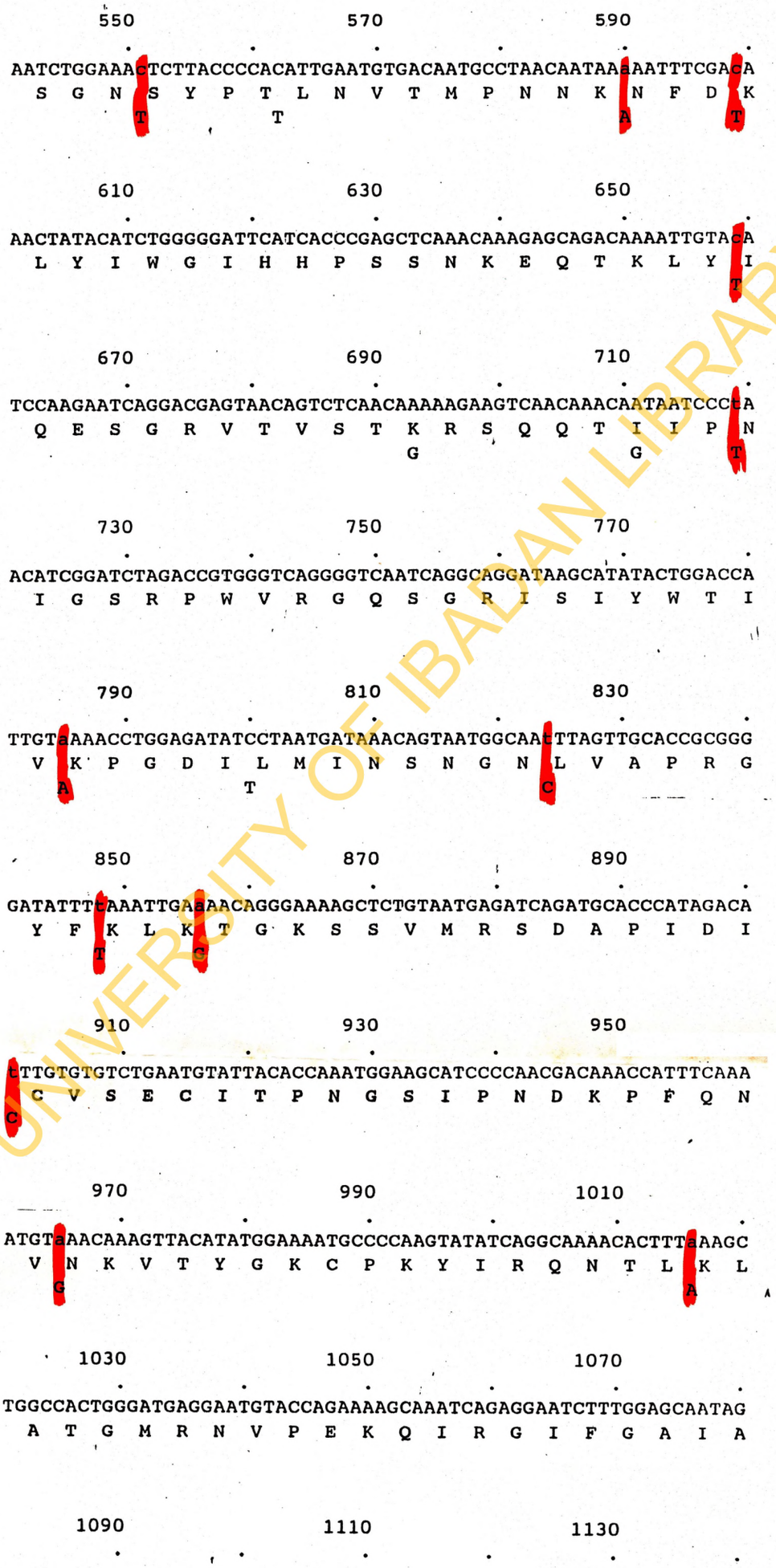
```
        10                  30                  50
---------+---------+---------+---------+---------+---------+
AGCAAAAGCAGGGGATATTTCTGTCAATCATGAAGACAACCATTGTTTTGATACTACTGA
                                M  K  T  T  I  V  L  I  L  L  T

        70                  90                  110
---------+---------+---------+---------+---------+---------+
CCCATTGGGTCTACAGTCAAAACCCAACCAGTGGCAACAACACAGCCACATTATGTCTGG
  H  W  V  Y  S  Q  N  P  T  S  G  N  N  T  A  T  L  C  L  G

       130                 150                 170
---------+---------+---------+---------+---------+---------+
GACACCATGCAGTAGCAAATGGAACACTGGTAAAAACAATAACTGATGACCAGATTGAGG
  H  H  A  V  A  N  G  T  L  V  K  T  I  T  D  D  Q  I  E  V

       190                 210                 230
---------+---------+---------+---------+---------+---------+
TGACAAATGCTACTGAATTAGTTCAGAGCATTTCAATAGGGAAAATATGCAACAACTCAT
  T  N  A  T  E  L  V  Q  S  I  S  I  G  K  I  C  N  N  S  Y

       250                 270                 290
---------+---------+---------+---------+---------+---------+
ATAGGGTTCTAGATGGAAGAAATTGCACATTAATAGATGCAATGCTAGGAGACCCCCACT
  R  V  L  D  G  R  N  C  T  L  I  D  A  M  L  G  D  P  H  C

       310                 330                 350
---------+---------+---------+---------+---------+---------+
GTGATGTTTTTCAGTATGAGAATTGGGACCTCTTCATAGAAAGAAGCAGCGCTTTCAGCA
  D  V  F  Q  Y  E  N  W  D  L  F  I  E  R  S  S  A  F  S  N

       370                 390                 410
---------+---------+---------+---------+---------+---------+
ATTGCTACCCATATGACATCCCTGACTATGCATCGCTCCGGTCCATTGTAGCATCCTCAG
  C  Y  P  Y  D  I  P  D  Y  A  S  L  R  S  I  V  A  S  S  G

       430                 450                 470
---------+---------+---------+---------+---------+---------+
GAACATTAGAATTCACAGCAGAGGGATTCACATGGACAGGTGTCACTCAAAACGGAAGAA
                                                  C
  T  L  E  F  T  A  E  G  F  T  W  T  G  V  T  Q  N  G  R  S
                                                  T

       490                 510                 530
---------+---------+---------+---------+---------+---------+
GTGGAGCCTGCAAAAGGGGATCAGCCGATAGTTTCTTTAGCCGACTGAATTGGCTAACAA
  G  A  C  K  R  G  S  A  D  S  F  F  S  R  L  N  W  L  T  K

       550                 570                 590
---------+---------+---------+---------+---------+---------+
AATCTGGAAATTCTTACCCCATATTGAATGTGACAATGCCTAAAAATAAAAATTTCGATA
                    C
  S  G  N  S  Y  P  I  L  N  V  T  M  P  K  N  K  N  F  D  K
                    T

       610                 630                 650
---------+---------+---------+---------+---------+---------+
AACTATACATCTGGGGGATTCATCACCCGAGCTCAAACAAAGAGCAGACAAAATTGTATA
  L  Y  I  W  G  I  H  H  P  S  S  N  K  E  Q  T  K  L  Y  I

       670                 690                 710
---------+---------+---------+---------+---------+---------+
TCCAAGAATCAGGACGAGTAACAGTCTCAACAGAAAGAAGTCAACAAACAGTAATCCCTA
```

```
  Q  E  S  G  R  V  T  V  S  T  E  R  S  Q  Q  T  V  I  P  N

       730                 750                 770
---------+---------+---------+---------+---------+---------+
ACATCGGATCTAGACCGTGGGTCAGGGGTCAATCAGGCAGGATAAGCATATACTGGACCA

  I  G  S  R  P  W  V  R  G  Q  S  G  R  I  S  I  Y  W  T  I

       790                 810                 830
---------+---------+---------+---------+---------+---------+
TTGTAAAACCTGGAGATATTCTAATGATAAACAGTAATGGCAACTTAGTTGCACCGCGGG

  V  K  P  G  D  I  L  M  I  N  S  N  G  N  L  V  A  P  R  G

       850                 870                 890
---------+---------+---------+---------+---------+---------+
GATATTTTAAATTGAGAACAGGGAAAAGCTCTGTAATGAGATCAGATGCACCCATAGACA

  Y  F  K  L  R  T  G  K  S  S  V  M  R  S  D  A  P  I  D  T

       910                 930                 950
---------+---------+---------+---------+---------+---------+
CTTGTGTGTCTGAATGTATTACACCAAATGGAAGCATCCCCAACGACAAACCATTCCAAA

  C  V  S  E  C  I  T  P  N  G  S  I  P  N  D  K  P  F  Q  N

       970                 990                1010
---------+---------+---------+---------+---------+---------+
ATGTGAACAAAGTTACATATGGAAAATGCCCCAAGTATATCAGGCAAAACACTTTAAAGC

  V  N  K  V  T  Y  G  K  C  P  K  Y  I  R  Q  N  T  L  K  L

      1030                1050                1070
---------+---------+---------+---------+---------+---------+
TGGCCACTGGGATGAGGAATGTACCAGAAAAGCAAATCAGAGGAATCTTTGGAGCAATAG

  A  T  G  M  R  N  V  P  E  K  Q  I  R  G  I  F  G  A  I  A

      1090                1110                1130
---------+---------+---------+---------+---------+---------+
CGGGATTCATAGAAAACGGCTGGGAAGGAATGGTTGATGGGTGGTATGGATTCCGATATC

  G  F  I  E  N  G  W  E  G  M  V  D  G  W  Y  G  F  R  Y  Q

      1150                1170                1190
---------+---------+---------+---------+---------+---------+
AAAATTCGGAAGGAACAGGACAAGCTGCAGATCTAAAGAGCACTCAAACAGCCATCGACC

  N  S  E  G  T  G  Q  A  A  D  L  K  S  T  Q  T  A  I  D  Q

      1210                1230                1250
---------+---------+---------+---------+---------+---------+
AGATCAATGGAAAATTAAACAGAGTGATTGAAAGGACCAATGAGAAATTCCATCAAATAG

  I  N  G  K  L  N  R  V  I  E  R  T  N  E  K  F  H  Q  I  E

      1270                1290                1310
---------+---------+---------+---------+---------+---------+
AGAAGGAATTCTCAGAAGTAGAAGGGAGAATCCAGGATTTGGAGAAGTATGTAGAAGACA

  K  E  F  S  E  V  E  G  R  I  Q  D  L  E  K  Y  V  E  D  T

      1330                1350                1370
---------+---------+---------+---------+---------+---------+
CCAAAATAGACCTATGGTCCTACAATGCAGAATTGCTGGTGGCTCTAGAAAATCAACATA

  K  I  D  L  W  S  Y  N  A  E  L  L  V  A  L  E  N  Q  H  T

      1390                1410                1430
---------+---------+---------+---------+---------+---------+
CAATTGACTTAACAGATGCAGAAATGAATAAATTATTCGAGAAGACTAGGCGCCAGTTAA

  I  D  L  T  D  A  E  M  N  K  L  F  E  K  T  R  R  Q  L  R

      1450                1470                1490
```

```
---------+---------+---------+---------+---------+---------+
GAGAAAACGCGGAAGACATGGGAGGTGGATGTTTCAGGATTTACCACAAATGTGATAATG

  E  N  A  E  D  M  G  G  G  C  F  R  I  Y  H  K  C  D  N  A

      1510                1530                1550
---------+---------+---------+---------+---------+---------+
CATGCATTGGATCAATAAGAAATGGGACATATGACCATTACATATACAGAGATGAAGCAT

  C  I  G  S  I  R  N  G  T  Y  D  H  Y  I  Y  R  D  E  A  L

      1570                1590                1610
---------+---------+---------+---------+---------+---------+
TAAACAACCGATTTCAAATCAAAGGTGTTGAGTTGAAATCAGGCTACAAAGATTGGATAC

  N  N  R  F  Q  I  K  G  V  E  L  K  S  G  Y  K  D  W  I  L

      1630                1650                1670
---------+---------+---------+---------+---------+---------+
TGTGGATTTCATTCGCCATATCATGCTTCTTAATTTGCGTTGTTCTATTGGGTTTCATTA

  W  I  S  F  A  I  S  C  F  L  I  C  V  V  L  L  G  F  I  M

      1690                1710                1730
---------+---------+---------+---------+---------+---------+
TGTGGGCTTGCCAAAAAGGCAACATCAGATGCAACATTTGCATTTGAGTAAACTGATAGT

  W  A  C  Q  K  G  N  I  R  C  N  I  C  I

      1750
---------+---------+--
TAAAAACACCCTTGTTTCTACT
```

(C) Nucleotide and amino acid sequences of A/Eq/Ibadan/9/91 (Ib9) and A/Eq/Ibadan/6/91 (Ib6) with nucleotide differences highlighted in orange.

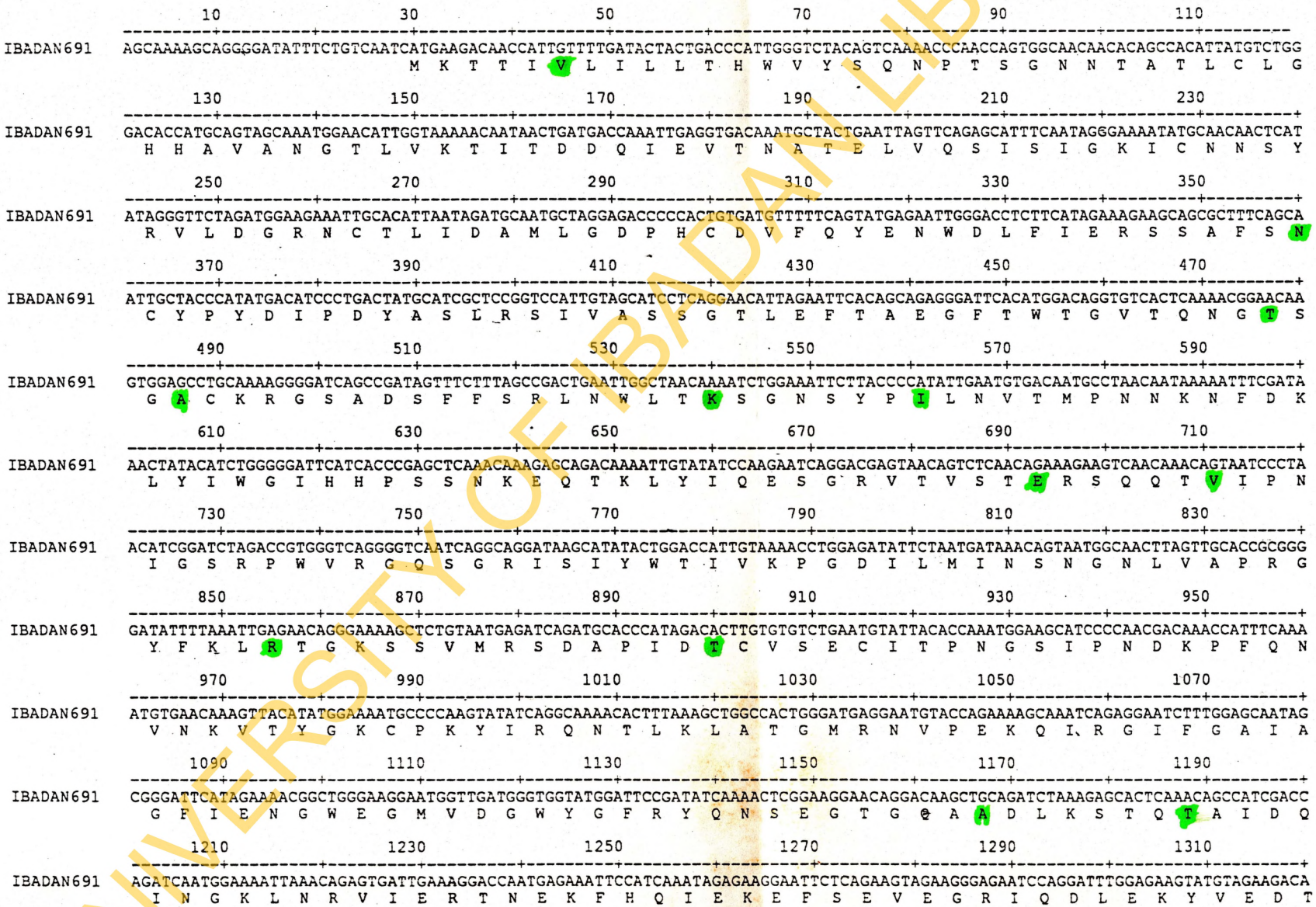
```
        10                  30                  50
         .         .         .         .         .         .
NNNNNNNNNNNGGGATATTTCTGTCAATCATGAAGACAACCATTATTTTGATACTACTGA
                                  M  K  T  T  I  I  L  I  L  L  T
AGCAAAAGCAG                                   G

        70                  90                 110
         .         .         .         .         .         .
CCCATTGGGTCTACAGTCAAAAtCCAACCAGTGGCAACAACACAGCCACATTATGTCTGG
  H  W  V  Y  S  Q  N  P  T  S  G  N  N  T  A  T  L  C  L  G
                       C

       130                 150                 170
         .         .         .         .         .         .
GACACCATGCAGTAGCAAATGGAACATTGGTAAAAACAATAACTGATGACCAAATTGAGG
  H  H  A  V  A  N  G  T  L  V  K  T  I  T  D  D  Q  I  E  V

       190                 210                 230
         .         .         .         .         .         .
TGACAAATGCTACTGAATTAGTTCAGAGCATTTCAATAGGGAAAATATGCAACAACTCAT
  T  N  A  T  E  L  V  Q  S  I  S  I  G  K  I  C  N  N  S  Y

       250                 270                 290
         .         .         .         .         .         .
ATAGaGTTCTAGATGGAAGAAATTGCACATTAATAGATGCAATGCTAGGAGACCCCCACT
  R  V  L  D  G  R  N  C  T  L  I  D  A  M  L  G  D  P  H  C
   G

       310                 330                 350
         .         .         .         .         .         .
GTGATGTCTTTCaGTATGAGAATTGGGACCTCTTCATAGAAAGAAGCAGCGCTTTCAGCA
  D  V  F  Q  Y  E  N  W  D  L  F  I  E  R  S  S  A  F  S  S
      T     A

       370                 390                 410
         .         .         .         .         .         .
gTTGCTACCCATATGACATCCCTGACTATGCATCGCTCCGGTCcATTGTaGCATCCTCAG
  C  Y  P  Y  D  I  P  D  Y  A  S  L  R  S  I  V  A  S  S  G
A                                        C     A

       430                 450                 470
         .         .         .         .         .         .
GAACATTgGAATTCACAGCAGAGGGATTCACATGGACAGGTGTCACTCAAAACGGAAGAA
  T  L  E  F  T  A  E  G  F  T  W  T  G  V  T  Q  N  G  R  S
      A                                                  C

       490                 510                 530
         .         .         .         .         .         .
GTGGAtCCTGCAAAAGGGGATCAGCCGATAGTTTCTTTAGCCGACTGAATTGGCTAACAg
  G  S  C  K  R  G  S  A  D  S  F  F  S  R  L  N  W  L  T  E
    G                                                       A
```



180g

```
CGGGATTCATAGAAAACGGCTGGGAAGGAATGGTTGATGGGTGGTATGGATTCCGATATC
  G  F  I  E  N  G  W  E  G  M  V  D  G  W  Y  G  F  R  Y  Q

      1150                1170                1190
       .         .         .         .         .         .
AAAACTCGGAAGGAACAGGACAAGCTGGAGATCTAAAGAGCACTCAAGCAGCCATCGACC
  N  S  E  G  T  G  Q  A  G  D  L  K  S  T  Q  A  A  I  D  Q
                      C                   A

      1210                1230                1250
       .         .         .         .         .         .
AGATCAATGGAAAATTAAACAGAGTGATTGAAAGGACCAATGAGAAATTCCATCAAATAG
  I  N  G  K  L  N  R  V  I  E  R  T  N  E  K  F  H  Q  I  E

      1270                1290                1310
       .         .         .         .         .         .
AGAAGGAATTtTCAGAAGTAGAAGGGAGAATCCAGGACTTGGAGAAGTATGTAGAAGACA
  K  E  F  S  E  V  E  G  R  I  Q  D  L  E  K  Y  V  E  D  T
           C                         T

      1330                1350                1370
       .         .         .         .         .         .
CtAAAATAGACCTATGGTCCTACAATGCAGAATTGCTGGTGGCTCTAGAAAATCAACATA
 K  I  D  L  W  S  Y  N  A  E  L  L  V  A  L  E  N  Q  H  T
C

      1390                1410                1430
       .         .         .         .         .         .
CAATTGACTTAACAGATGCAGAAATGAATAAATTATTCGAGAAGACTAGaCGCCAGTTAA
  I  D  L  T  D  A  E  M  N  K  L  F  E  K  T  R  R  Q  L  R
                                                G

      1450                1470                1490
       .         .         .         .         .         .
GAGAAAACGCGGAAGACATGGGAGGTGGATGTTTCAAGATTTACCACAAATGTGATAATG
  E  N  A  E  D  M  G  G  G  C  F  K  I  Y  H  K  C  D  N  A

      1510                1530                1550
       .         .         .         .         .         .
CATGCATTGGATCAATAAGAAATGGGACATATGACCATTACATATACAGAGATGAAGCAT
  C  I  G  S  I  R  N  G  T  Y  D  H  Y  I  Y  R  D  E  A  L

      1570                1590                1610
       .         .         .         .         .         .
TAAACAACCGgTTTCAAATCAAAGGTGTTGAGTTGAAATCAGGCTACAAAGATTGGATAC
  N  N  R  F  Q  I  K  G  V  E  L  K  S  G  Y  K  D  W  I  L
           A        C

      1630                1650                1670
       .         .         .         .         .         .
TGTGGATTTCATTCGCCATATCATGCTTCTTAATTTGCGTTGTTCTATTGGGTTTCATTA
  W  I  S  F  A  I  S  C  F  L  I  C  V  V  L  L  G  F  I  M
```

(B) Nucleotide and amino acid sequences of A/Eq/Ibadan/6/91. Amino acid differences with A/Eq/Ibadan/9/91 are shown in green.



180d

```
                1330                1350                1370                1390                1410                1430
                ----------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
IBADAN691       CCAAAATAGACCTATGGTCCTACAATGCAGAATTGCTGGTGGCTCTAGAAAATCAACATACAATTGACTTAACAGATGCAGAAATGAATAAATTATTCGAGAAGACTAGGCGCCAGTTAA
                  K  I  D  L  W  S  Y  N  A  E  L  L  V  A  L  E  N  Q  H  T  I  D  L  T  D  A  E  M  N  K  L  F  E  K  T  R  R  Q  L  R

                1450                1470                1490                1510                1530                1550
                ----------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
IBADAN691       GAGAAAACGCGGAAGACATGGGAGGTGGATGTTTCAAGATTTACCACAAATGTGATAATGCATGCATTGGATCAATAAGAAATGGGACATATGACCATTACATATACAGAGATGAAGCAT
                  E  N  A  E  D  M  G  G  G  C  F  K  I  Y  H  K  C  D  N  A  C  I  G  S  I  R  N  G  T  Y  D  H  Y  I  Y  R  D  E  A  L

                1570                1590                1610                1630                1650                1670
                ----------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
IBADAN691       TAAACAACCGATTTCAAATCAAAGGTGTTGAGTTGAAATCAGGCTACAAAGATTGGATACTGTGGATTTCATTCGCCATATCATGCTTCTTAATTTGCGTTGTTCTATTGGGTTTCATTA
                  N  N  R  F  Q  I  K  G  V  E  L  K  S  G  Y  K  D  W  I  L  W  I  S  F  A  I  S  C  F  L  I  C  V  V  L  L  G  F  I  M

                1690                1710                1730                1750
                ----------+---------+---------+---------+---------+---------+---------+---------+--
IBADAN691       TGTGGGCTTGCCAAAAAGGCAACATCAGATGCAACATTTGCATTTGAGTAAACTGATAGTTAAAAACACCCTTGTTTCTACT
                  W  A  C  Q  K  G  N  I  R  C  N  I  C  I
```


180d

180h

```
      1690                1710                1730
       .                   .                   .                   .
TGTGGGCTTGCCAAAAAGGCAACATCAGATGCAACATTTGCATTTGAGTAAACTGATAGT
  W  A  C  Q  K  G  N  I  R  C  N  I  C  I

      1750
       .                   .
TAAAAACACCCTTGTTTCTACT
```


Legend to Fig. 17.

Nucleotide sequences are from the 3^1 end. The initiation codon is at Nucleotides 30 - 32, the non-coding region is 29 bases long from the 3^1 end and 38 bases long from the 5^1 end, the signal peptide is 14 amino acids long from nucleotides 33 - 74 at the 3^1 end, the HA1 reading frame starts at nucleotide 75 and terminates at nucleotide 1058 followed by the cleavage site which is 3 nucleotides long and the HA2 reading frame from nucleotides 1062 - 1725. The nucleotide differences in the 3 viruses are indicated. The first 12 terminal nucleotides from the 3^1 end are conserved in all influenza A viruses. Nucleotide sequences in the figure are complementary to the 3^1 end of vRNA which are cRNA positive sense sequences from the 5^1 end.

The nucleotide and amino acid sequences have been deposited at the EMBL culeotide database at the European Bioinformatics Institute, Hinxton, U.K. and have been given the accession numbers: A/equine/Ibadan/6/91, X95637 and A/equine/Ibadan/9/91, X95638.

RT/PCR and cycle sequencing method as described above. The PCR product was directly sequenced using 5′ terminal end labelled oligonucleotide primers. Although RNA and plasmid DNA sequencing was also carried out, in my experience, cycle sequencing of PCR products obviates the necessity to determine the nucleotide sequence of multiple DNA clones following PCR which is required with the alternative strategy of cloning of PCR products, since as with direct RNA sequencing, an average sequence is obtained so long as ample RNA was used to make the cDNA copy prior to PCR amplification.

The complete nucleotide sequences of the HA genes of the three Ibadan viruses are as shown in Fig. 17. Fig. 18 shows the complete nucleotide seguences of the HA genes of ten equine-2 (H3N8) viruses isolated over a 29-year period (1963-1991) including the original equine-2 prototype virus A/Eq/Miami/63, the prototypic Northern European strain A/Eq/Suffolk/89, the first equine-2 virus isolated in temperate North Africa A/Eq/Algeria/72 and the three Ibadan viruses designated Ib4, Ib6 and Ib9, respectively. Fig. 19 shows the complete amino acid sequences of the three Ibadan viruses compared with Suffolk/89 and consensus sequences from the database while Fig. 20 shows the amino acid differences in the HA1 of equine H3 influenza viruses isolated over a 29 year period between 1963 and 1991 including the Ibadan and Northern European viruses isolated between 1989 and 1991. The coding sequences of the three Ibadan viruses are 1695 nucleotides long and indicate gene base compositions rich in A (36.3-36.5%) and average in the other three bases G, T, C (20-23.8%).

The signal peptides of the three viruses and those of Suffolk/89 and Consensus sequence are 14 amino acids long in contrast to those of equine H7 viruses which are 18

amino acids long but show a very high homology (92.8%) for Ib4 and Ib6 and 100% for Ib9, Suffolk/89 and Consensus sequence in contrast to equine H7 viruses with about 50% homology in their signal peptide sequence (Gibson *et al.*, 1988). The HA1 of Ibadan viruses is 329 amino acids long with an arginine (R) at the carboxyl terminus. The HA1 of Ib4 and Ib6 contains 8 N-linked (asparagin-linked) glycosylation sites at positions 8, 22, 38, 53, 63, 133, 165 and 285 while the HA1 of Ib9 contains 7 N-linked glycosylation sites at positions 8, 22, 38, 53, 63 and 285. The HA2 of the three virsues contains only one N-linked glycosylation site at position 154 of the HA2 polypeptide. In comparison with the original equine-2 prototype virus (Miami/63), the Ibadan viruses showed a number of nucleotide (base) changes. Ib4 and Ib6 showed 122 base changes while Ib9 showed 132 base changes indicating a considerable drift in the Ibadan viruses from the original prototype virus. This represents a mutation (nucleotide substitution) rate of 0.24% per year for Ib4 and Ib6 and 0.26% per year for Ib9. These base changes were at position 43 (C-T), 45 (A-G) for Ib4 and Ib6, 71 (C-T), 72 (C-T), 90 (G-A), 111 (C-T), 113 (G-A), 116 (C-T), 147 (C-T), for Ib9, 173 (G-A) for Ib9, 211 (C-T), 216 (C-T), 237 (C-T), 249 (C-T), 263 (C-T), 287 (G-A), 293 (T-C), 299 (T-C), 301 (A-G), 338 (T-A), 350 (T-C), 378 (G-A), 404 (T-C), 405 (C-A), 410 (G-A), 416 (C-T), 425 (G-A), 428 (A-G), for Ib9, 436 (T-C), 437 (G-A), 477 (G-A), 478 (G-C), for Ib6, 483 (A-G), 485 (C-A), 486 (G-T), for Ib9, 493 (G-A), 540 (C-A) for Ib4 and Ib6, C-G for Ib9, 547 (A-G), 550 (G-A), 551 (T-C) for Ib9, 554 (C-T), 562 (C-T) for Ib4, 584 (C-A), 590 (C-A) for Ib4 and

Ib6, 620 (C-T), 631 (T-C), 632 (T-C), 633 (A-T), 638 (T-C), 641 (T-A), 659 (T-C), for Ib9, 660 (G-A), 667 (C-A), 674 (G-A), 686 (T-C), 693 (A-G), for Ib4 and Ib6, 710 (G-A), 711 (A-G), for Ib4 and Ib6, 719 (C-T), 728 (G-A), 731 (C-T), 773 (T-C), 785 (G-A), 798 (G-A), 800 (C-T), for Ib4 and Ib6, 828 (A-G), 839 (A-G), 848 (C-T) for Ib4 and Ib6, 852 (A-T), 855 (C-A), 856 (G-A), for Ib9, 857 (G-A), 863 (A-G), 873 (A-G), 914 (G-A), 953 (G-A), 956 (T-C), 969 (A-C) for Ib9, 971 (G-A), 999 (G-A), 1003 (G-A) for Ib9, 1025 (T-C), 1028 (C-T), 1097 (T-C), 1106 (G-A) for Ib9, 1142 (G-A), 1145 (T-C) for Ib9, 1154 (G-A), 1158 (C-G), 1159 (T-G), 1160 (G-A), 1168 (C-G) for Ib9, 1188 (G-A) for Ib4 and Ib6, 1196 (T-C), 1206 (A-C) for Ib9, 1211 (G-A), 1216 (G-A), 1232 (G-A), 1234 (A-G), 1235 (A-G), 1238 (T-C), 1244 (A-G), 1250 (T-C), 1271 (C-T) for Ib9, 1295 (A-G), 1298 (C-T) for Ib4 and Ib6, 1310 (C-T), 1322 (C-T) for Ib9, 1346 (C-T), 1352 (G-A0), 1355 (A-G), 1361 (A-G), 1364 (C-T), 1382 (G-A), 1389 (C-T), 1403 (G-A), 1426 (C-T), 1430 (G-A) for Ib9, 1463 (G-A), 1464 (A-G), 1465 (A-G), 1472 (A-G), 1484 (T-C), 1510 (A-G), 1539 (G-T), 1556 (G-A), 1571 (G-A), for Ib4 and Ib6, 1580 (T-C), 1582 (G-A), and 1709 (G-A). About two-thirds of these changes occurred in the HA1 domain of the HA. Compared with the prototypic Northen European strain, Suffolk/89, the three Ibadan viruses showed changes at 17, 18, and 20 bases respectively, at positions 45, 147, 173, 263, 361, 428, 478, 486, 540, 551, 562, 589, 590, 641, 659, 805, 848, 956, 968, 1002, 1003, 1017, 1019, 1028, 1097, 1098, 1145, 1188, 1206, 1271, 1298, 1322, 1430, 1478 and 1571. The three viruses also showed variation among themselves. Ib4 differed from Ib6 at 3 bases at positions 478, 562 and 486 indicating a high degree of homology between these two viruses, while both of them differed from Ib9 at about 25-27

bases at positions 45, 83. 245, 308, 361, 428, 478, 486, 540, 551, 562, 599, 658, 693, 711, 800, 824, 856, 901, 965, 1168, 1188, 1271, 1298, 1322, 1430 and 1571 (Table 14). Fifteen of these base changes were synonymous (silent) while 12 led to amino acid substitutions. One of these changes was located in the signal peptide and two were in the HA2 domain. All but one of the amino acid substitutions in Ib4 and Ib6 were located in or close to each of the antigenic sites of the HA with three changes seen in site A, two in site B, one in site C, one in site D and one at the junction of sites D and E. The other amino acid change in the HA1 outside the antigenic sites (residue 213) is buried in the HA trimer. The HA nucleotide sequence analysis clearly showed a close relationship between the Ibadan viruses and those that were isolated in Europe between 1989 and 1991 and in Hong Kong in 1992: Ib4 and Ib6 are very closely related to the prototype Northern European strain, Suffolk/89 to which Hong Kong/92 virus is also closely related while Ib9 is most closely related to Arundel/12369/91 isolated in UK in 1991 (Table 14). Only the region of the genome encoding the signal peptide, HA1 and the amino-terminal 40 amino acids of HA2 of Arundel/12369/91 virus has been determined (Lindstrom *et al.*, 1994). There are three base changes in that region of the genome between Arundel/12369/91 and Ibadan/9/91: two at positions 83(T-C) and 824(T-C) are synonymous, encoding the same amino acid residue and one at position 943(A-G) encodes an amino acid substitution (N to S at residue 290) in antigenic site C.

Base changes resulted in amino acid changes in HA1 domain of the three viruses while a base change resulted in amino acid change in the HA2 domain (Figs. 17 to 20). The base changes in Ib4 and Ib6 at nucleotides 45 (A-G), 478 (G-C), 562 (T-C) and 805

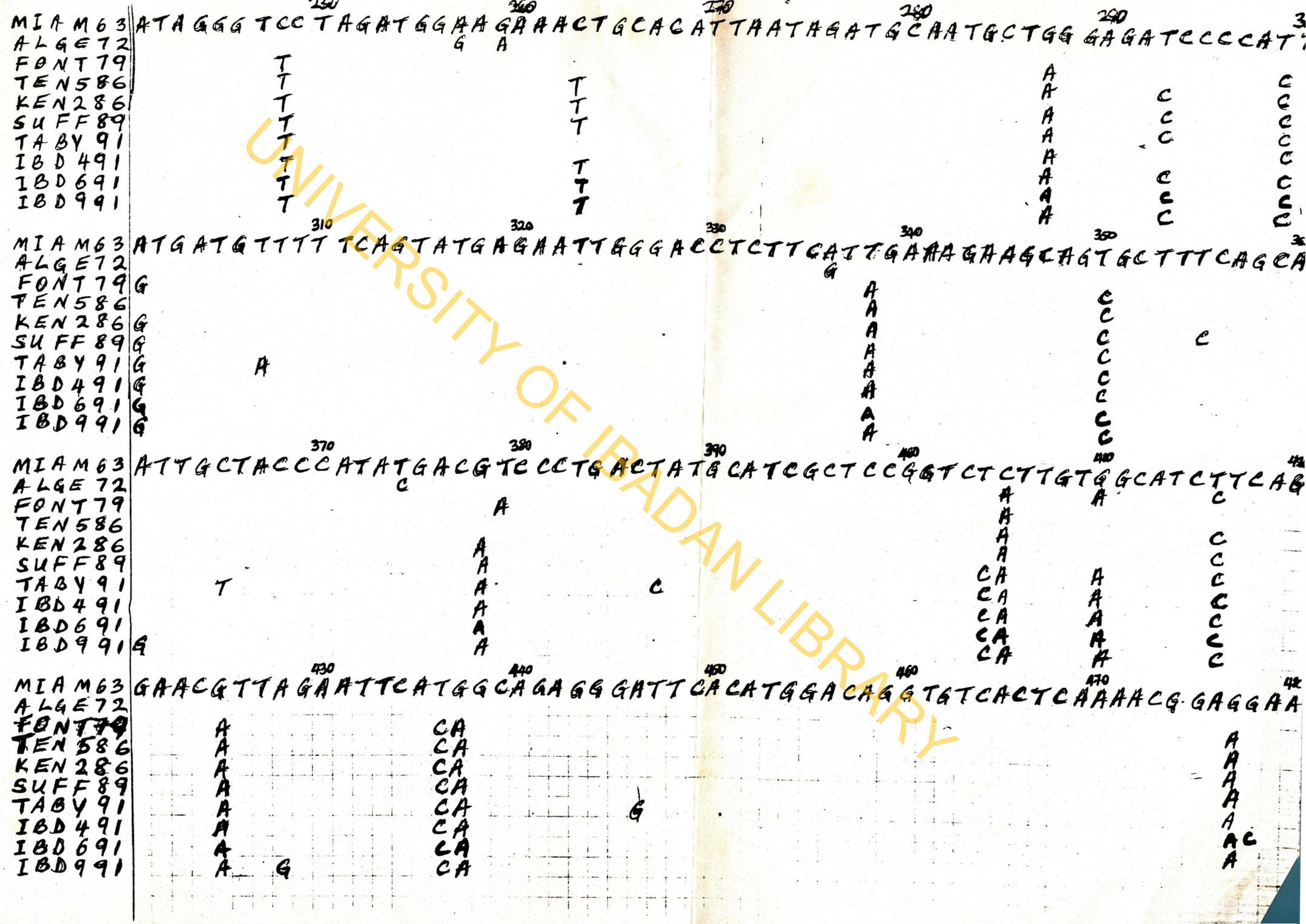
(C-T) in the HA1 domain and 1188 (G-A) in the HA2 domain resulted in amino acid changes at residues 6 from I to V (isoleucine to valine) which is within the signal sequence (McCauley *et al.*, 1979, McCauley, 1987), residue 135' from R to T (arginine to threonine), residue 178 from I to T (isoleucine to threonine) and residue 244 from T (arginine to methionine) and residue 43 from A to T (alanine to threonine) in HA2 domain. In Ib9, the changes at nucleotide 361 (A-G) resulted in amino acid change at residue 96 from N to S (asparagine to serine), at nucleotide 486 (G-T) resulted in amino acid change at residue 138 from A to S (arginine to serine), and at nucleotide 540 (A-G) resulted in amino acid change at residue 156 from K to E (lysine to glutamic acid), at nucleotide 856 (G-A) resulted in amino acid change at residue 261 from R to K (arginine to lysine) in HA1 and nucleotide 1188 (A-G) resulted in amino acid change at residue 43 from A to T (alanine to threonine) in HA2 domain. The change in the HA2 domain is unique to Ib4 and Ib6 viruses. As shown in Plate 9, these amino acid changes in Ib4 and Ib6 mapped to the signl sequence and antigenic sites A, C and D respectively, while in Ib9, the changes mapped to antigenic sites A, B, C and D, respectively. These nucleotide and amino acid differences in the three Ibadan viruses occured in the functional domain of the HA and are a reflection of the antigenic diversity exhibited by the viruses in their antigenic cross-reactivity with Mabs and polyclonal antisera seen in Chapter 2, where Ib4 was also observed to be very close to Ib6. The differences in nucleotide sequences between the Ibadan viruses and the prototypic Northern European virus Suffolk/89 also resulted in amino acid changes at residues 6, 150, 178 and 259 which also mapped to antigenic sites E, B, C and D, respectively. Fig. 21 shows the amino acid codons and the

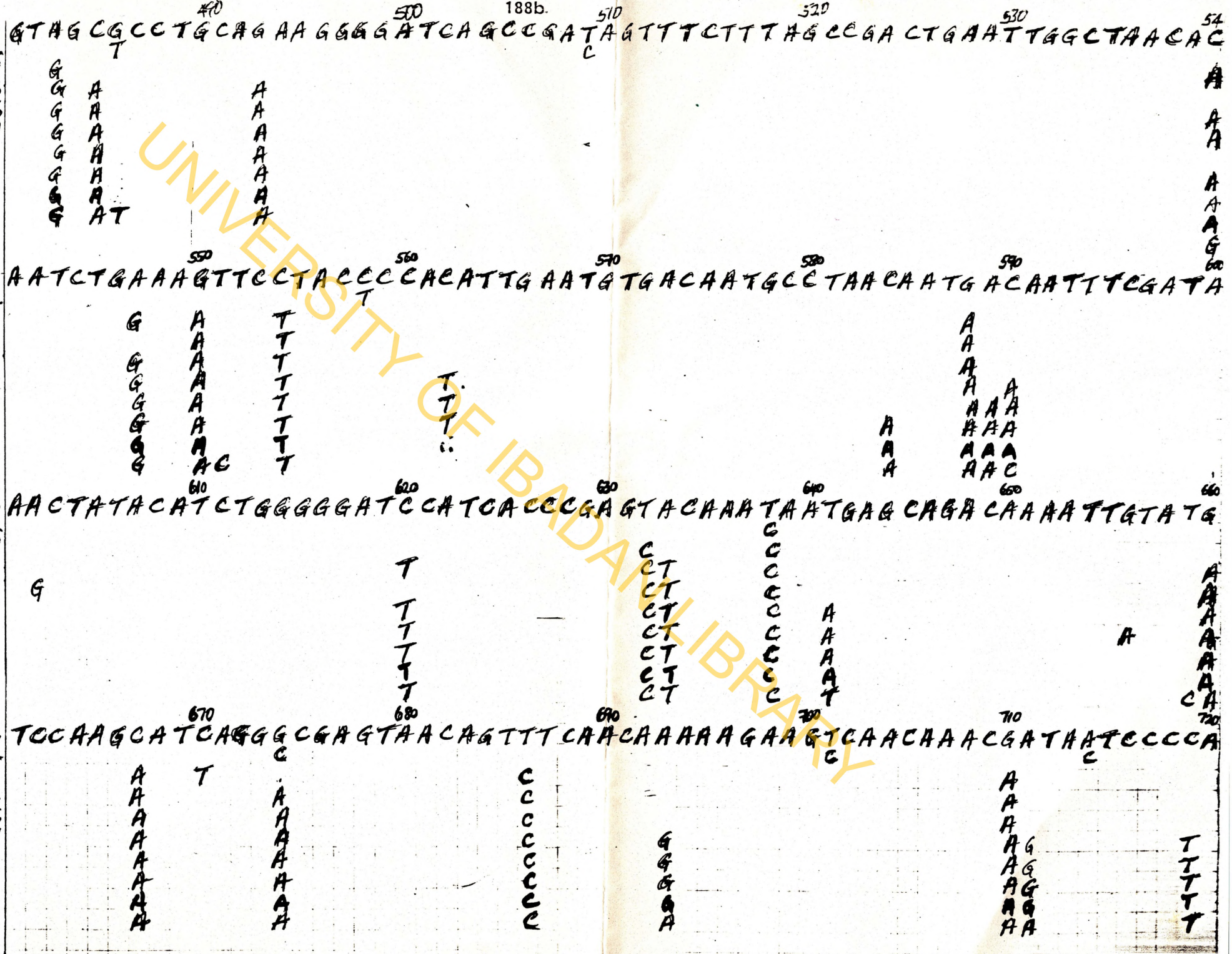
single letter codes for amino acids. Plate 9 shows the changes at the antigenic sites on one monomer of the equine H3 HA molecule for the Ibadan viruses as well as Suffolk/89 and Taby/91 described here as the 1989/93 cluster. The changes seen generally in the cluster viruses are shown in yellow color while the red color shows the changes that are unique to Ibadan viruses at antigenic site D in the HA2 domain. HA1 is light blue in that monomer, HA2 is dark blue while the other two monomers are also shown in light blue.

Table 14. Nucleotide sequence variation between UK and Nigerian equine influenza viruses.

Base Number	Suffolk 89 (Ref)	Ibadan691	Ibadan991	Arundel91 (Ref.)	Amino acid sequence change.	Remark
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 Suffolk89
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	

FIG. 18. NUCLEOTIDE SEQUENCES OF THE HA GENES OF TEN EQUINE H3 INFLUENZA VIRUSES. THE COMPLETE SEQUENCE OF MIAM/63 HA GENE IS WRITTEN IN FULL AND NUCLEOTIDE SUBSTITUTIONS OF OTHER VIRUSES ARE INDICATED.





```
                1450       1460       1470       1480       1490       1500
MIAM63 GAGAAAA CGCGGAAGACATGGGGAATGGATGTTTCAAGATTTATCACAAATGTGATAATG
ALGE72                 T                   C
FONT79
TEN586                    AGG                  C
KEN286                    AGG                  C
SUFF89                    AGG                  C
TABY91                    AGG                  C
IBD491                    AGG                  C
IBD691                    AGG                  C
IBD991                    AGG                  C

                1510       1520       1530       1540       1550       1560
MIAM63 CATGCATTGAATCAATAAGAAATGGGACATATGACCATGACATATACAGAGATGAGGCAT
ALGE72                                                  T
FONT79          G                  T                          A
TEN586          G                  T                          A
KEN286          G                  T                          A
SUFF89          G                  T                          A
TABY91          G                  T                          A
IBD491          G                  T                          A
IBD691          G                  T                          A
IBD991          G                  T                          A

                1570       1580       1590       1600       1610       1620
MIAM63 TAAACAACCGGTTTCAAATTAGAGGTGTTGAGTTGAAATCAGGCTACAAAGATTGGATAC
ALGE72                 C   A      A
FONT79          A         AA
TEN586          A         AA                 A   C
KEN286          A         AA
SUFF89          A      C  AA
TABY91          A      C  AA
IBD491          A      C  AA
IBD691          A      C  AA
IBD991          G      C  AA

                1630       1640       1650       1660       1670       1680
MIAM63 TGTGGATTTCATTCGCCATATCATGCTTCTTAATTTGCGTTGTTCTATTGGGTTTCATTA
ALGE72                             T                                C
FONT79
TEN586                      C
KEN286
SUFF89
TABY91
IBD491
IBD691
IBD991
```

1690 1700 1710 1720 1730 1740

MIAM163 TGTGGCTTGCCAAAAGGCAACATCAGGTGCAACATTTGCATTTGAGTAAACTGATAGT
ALGE72 A A
FONT79 A A
TEN586 A A
KEN286 A A
SUFF89 A A
TABY91 A A
IBD491 A A
IBD691 A A
IBD991 A A

1750 1760

MIAM63 TAAAAACACCCTTGTTTCTACT
ALGE72
FONT79
TEN586
KEN286
SUFF89
TABY91
IBD491
IBD691
IBD991

Fig. 19. Amino acid sequences of the HA genes of Ibadan viruses.

```
                1                                                   50
Suffolk89       i
Ibadan491
Ibadan691
Ibadan991       i
Concensus  MKTTIVLILL THWVYSQNPT SGNNTATLCL GHHAVANGTL VKTITDDQIE
                51                  → HA1                          100
Suffolk89
Ibadan491
Ibadan691
Ibadan991
Concensus  VTNATELVQS ISIGKICNNS YRVLDGRNCT LIDAMLGDPH CDVFQYENWD
                101                                                150
Suffolk89
Ibadan491                                                          t*
Ibadan691                                                          t*
Ibadan991               s
Concensus  LFIERSSAFS NCYPYDIPDY ASLRSIVASS GTLEFTAEGF TWTGVTQNGR
                151                                                200
Suffolk89
Ibadan491                                       k
Ibadan691
Ibadan991     s                 e       t
Concensus  SGACKRGSAD SFFSRLNWLT KSGNSYPILN VTMPNNKNFD KLYIWGIHHP
                201                                                250
Suffolk89
Ibadan491
Ibadan691                               g
Ibadan991                               i
Concensus  SSNKEQTKLY IQESGRVTVS TERSQQTVIP NIGSRPWVRG QSGRISIYWT
                251                                                300
Suffolk89 ---       t
Ibadan491
Ibadan691
Ibadan991                               k               i
Concensus  IVKPGDILMI NSNGNLVAPR GYFKLRTGKS SVMRSDAPID TCVSECITPN
                301                                                350
Suffolk89
Ibadan491
Ibadan691
Ibadan991
Concensus  GSIPNDKPFQ NVNKVTYGKC PKYIRQNTLK LATGMRNVPE KQIRGIFGAI
                351                             a   ← HA1  → HA2   400
Suffolk89
Ibadan491
Ibadan691
Ibadan991                                       a
Concensus  AGFIENGWEG MVDGWYGFRY QNSEGTGQAA DLKSTQTAID QINGKLNRVI
                401                                                450
Suffolk89
Ibadan491
Ibadan691
Ibadan991
Concensus  ERTNEKFHQI EKEFSEVEGR IQDLEKYVED TKIDLWSYNA ELLVALENQH
                451                                                500
Suffolk89
Ibadan491
Ibadan691
Ibadan991
Concensus  TIDLTDAEMN KLFEKTRRQL RENAEDMGGG CFKIYHKCDN ACIGSIRNGT
```

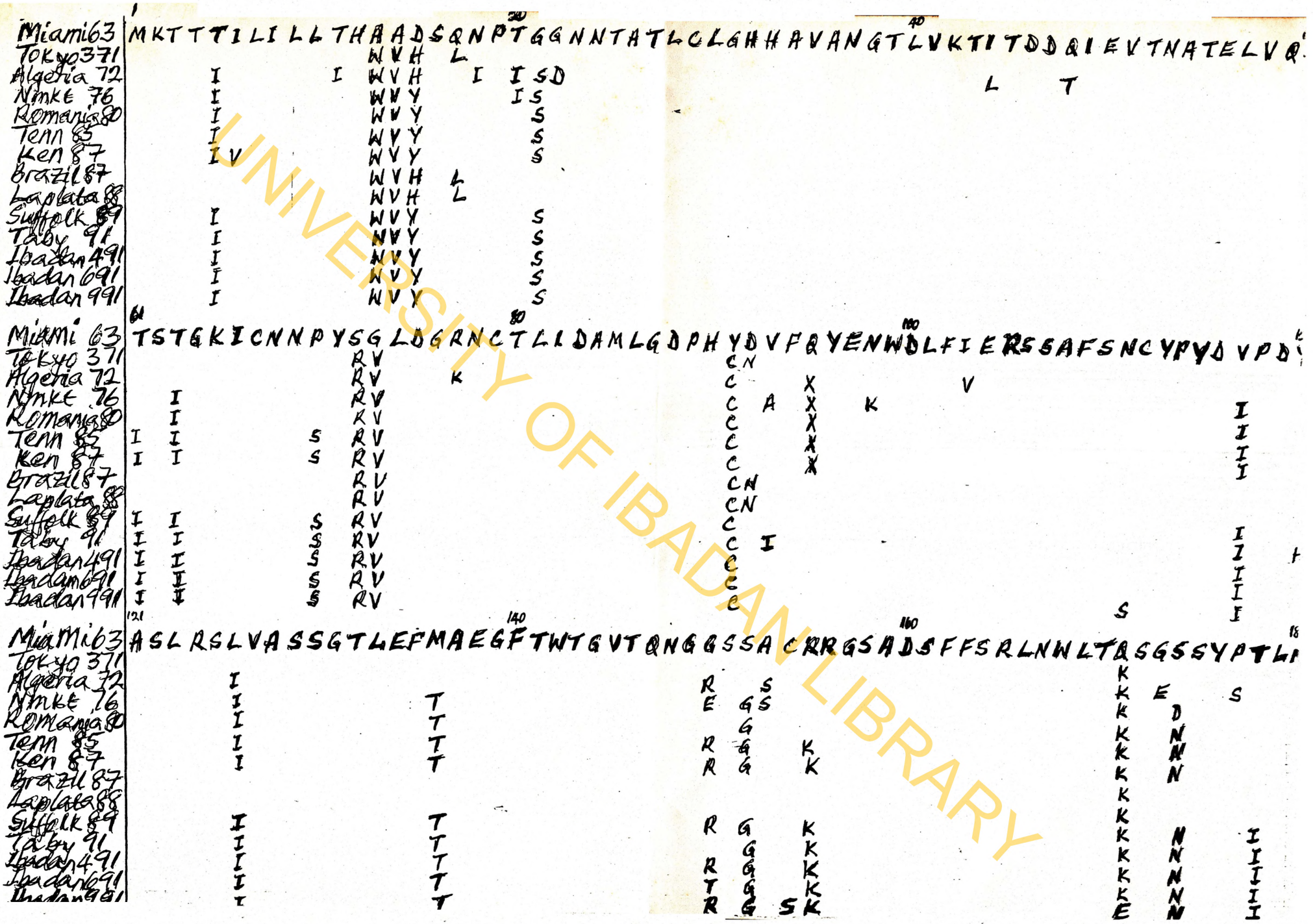
```
Suffolk89
Ibadan491
Ibadan691
Ibadan991
Concensus YDHYIYRDEA LNNRFQIKGV ELKSGYKDWI LWISFAISCF LICVVLLGFI
                551           565
Suffolk89
Ibadan491
Ibadan691
Ibadan991
Concensus MWACQKGNIR CNICI
```

Legend to Fig. 20

The sequences are compared with that of A/Eq/suffolk/89. All sequences are aligned to maximize homology with a concensus sequence from the database. Potential asparagine-linked (N-linked) glycosylation sites are the sequences N - X - S/T where X is any amino acid except aspartic acid or proline, they are underlined in the concensus sequence and the newly introduced glycosylation site at residue 133 due to amino acid change at residue 135 in Ib4 and Ib6 is highlighted with an asteric making 9 glycosylation sites for Ib4 and Ib6 and 8 sites for Ib9. The N-linked sites are NNT at position 8, NGT at position 22, NAT at position 38, NNS at position 53, NCT at position 63, NGT at position 133, NVT at position 165, NGS at position 285 in HA1 domain and NGT at position 154 in HA2 domain.

4.3.9 Phylogenetic analysis of the HA genes

Analysis of the three HA gene sequences and those of other equine H3 HAs have been carried out using the DNADIST program and a Neighbour-joining tree was made (Felsenstein, 1985, 1989, 1993) and the phylogenetic tree is shown in Fig. 22. The results from this analysis clearly showed a close relationship between the Ibadan viruses and those that were isolated in Europe in 1989 and 1991. This is reflected by comparison of the amino acid sequence changes seen between the Ibadan viruses and other equine H3 viruses. The differences in bases and amino acid sequences between the prototype-1989 (A/Eq/Suffolk/89) virus (Binus *et al.*, 1993), Arundel/12369/91 which is a representative virus from the second branch of 1989/93 cluster and the Ibadan viruses are shown in Figs. 18 & 19 and Table 14 respectively.



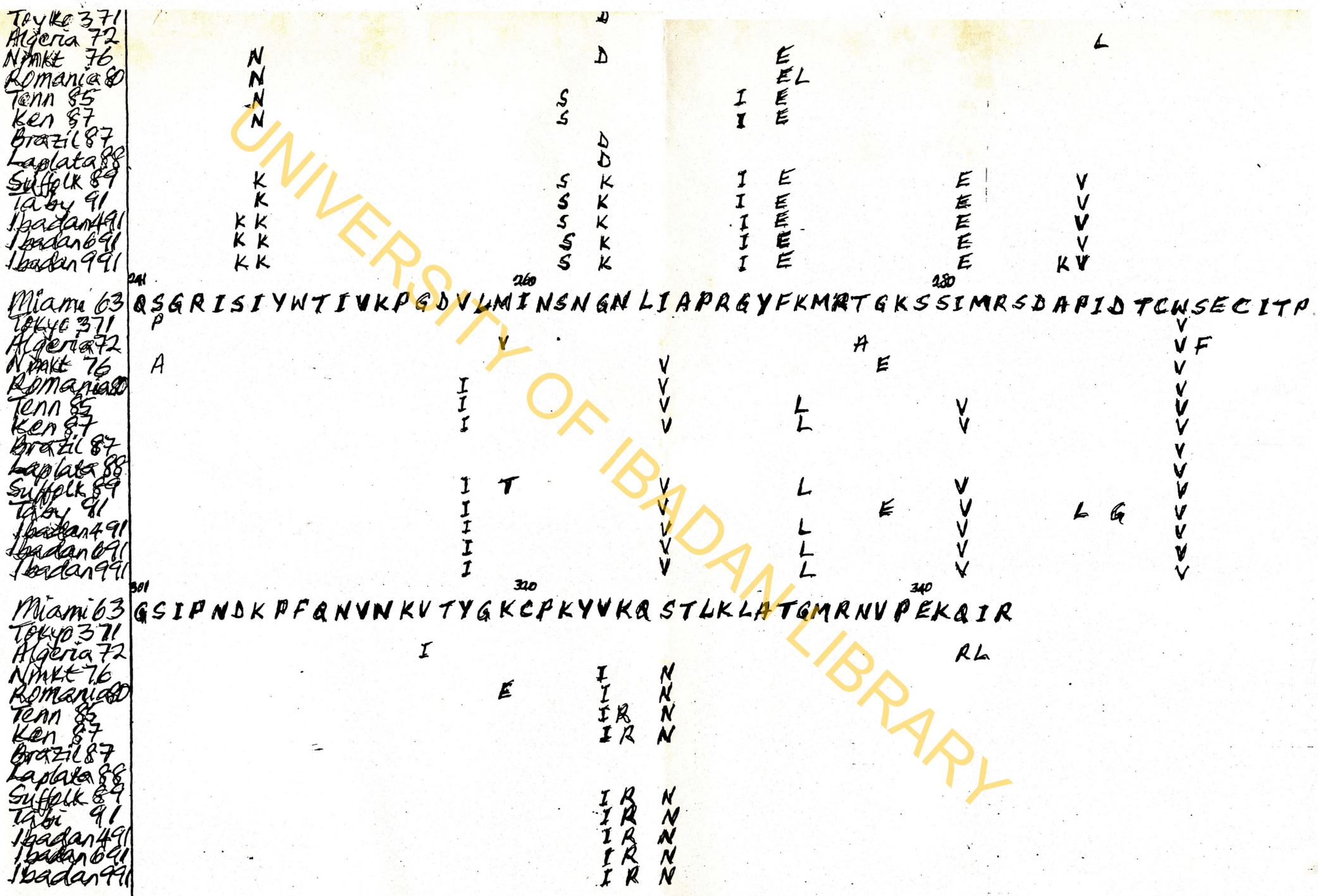


Fig. 21: Amino acid codons and the single letter codes for amino acids.

A = Alanine (Ala), 4 codons: GCA, GCC, GCG, GCT.
C = Cystine (Cys), 2 codons: TGT, TGC.
D = Aspartic acid (Asp), 2 codonsL GAT, GAC.
E = Glutamic acid (Glu), 2 codons: GAA, GAG.
F = Phenylalanine (Phe), 2 codons: TTC, TTT.
H = Histidine (His), 2 codons: GGA, GGC, GGG, GGT.
I = Isoleucine (Ile), 3 codons: ATA, ATC, ATT.
K = Lysine (Lys), 2 codons: AAA, AAG.
L = Leucine (Leu), 6 codons: CTA, CTG, CTT, CTC, TTA, TTG.
M = Methionine (Met), 2 codons: ATG (start), AUG (start).
N = Asparagine (Asn), 2 codons: AAC, AAT/AAU.
P = Proline (Pro), 5 codons: CCG, CCA, CCC, CCT, CCU.
Q = Glutamine (Gln), 2 codons: CAA, CAG.
R = Arginine (Arg), 6 codons: AGA, AGG, CGT, CGA, CGC, CGG/CGU.
S = Serine (Ser), 5 codons: AGC, AGT, TCA, TCT, TCC
T = Threonine (Thr), 4 codons: ACA, ACC, ACG, ACT/ACU
V = Valine (Val), 4 codons: 4 codons: GTT, GTA, GTG, GTC.
W = Tryptophan (Trp), 1 codon: TGG.
Y = Tyrosine (Tyr), 2 codons: TAC, TAT.

Start codon: ATG for DNA and AUG for RNA.
Stop codons: TAG, TAA, TGA.
Amber stop codon: Triplet TAG.

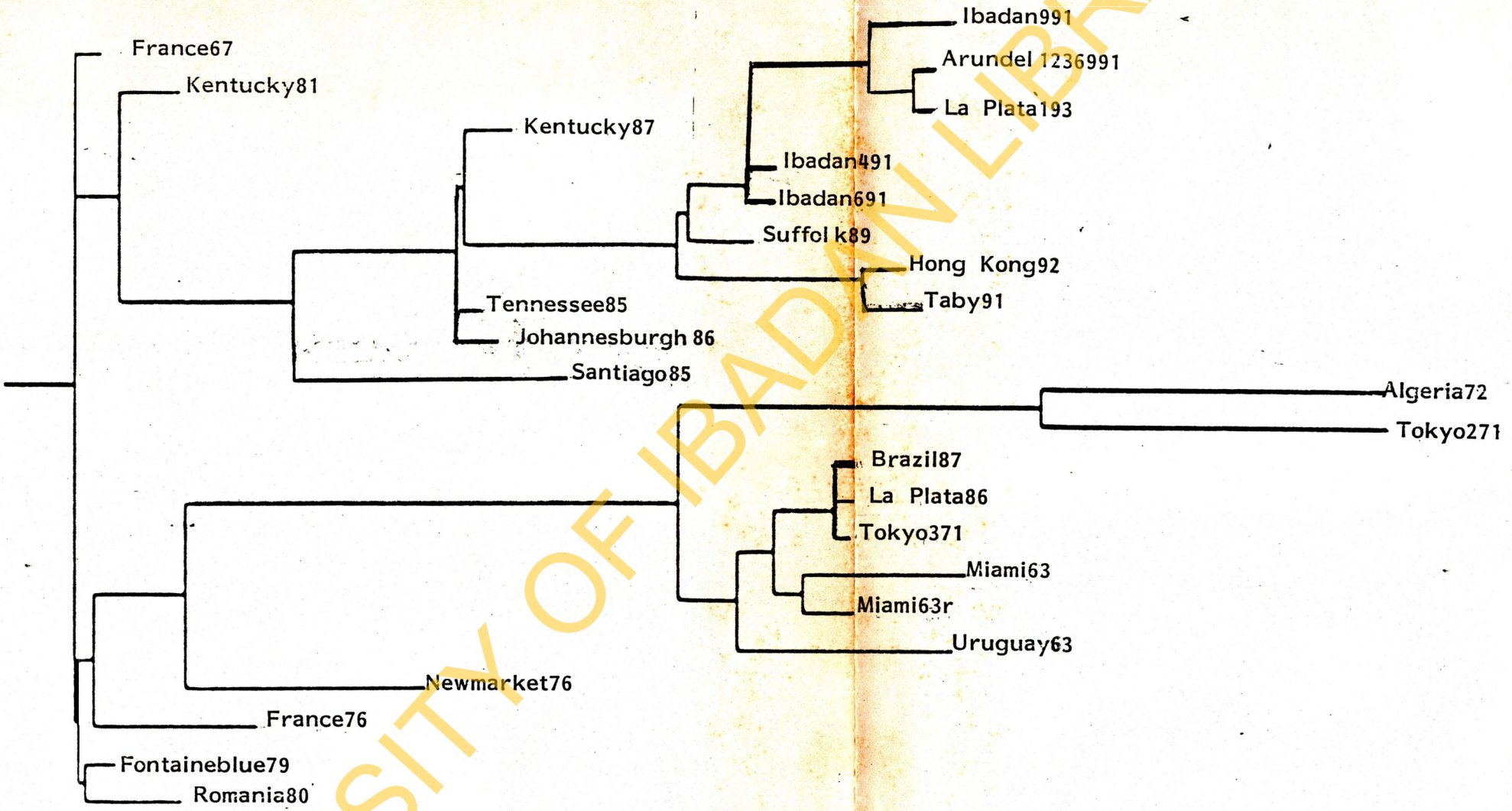
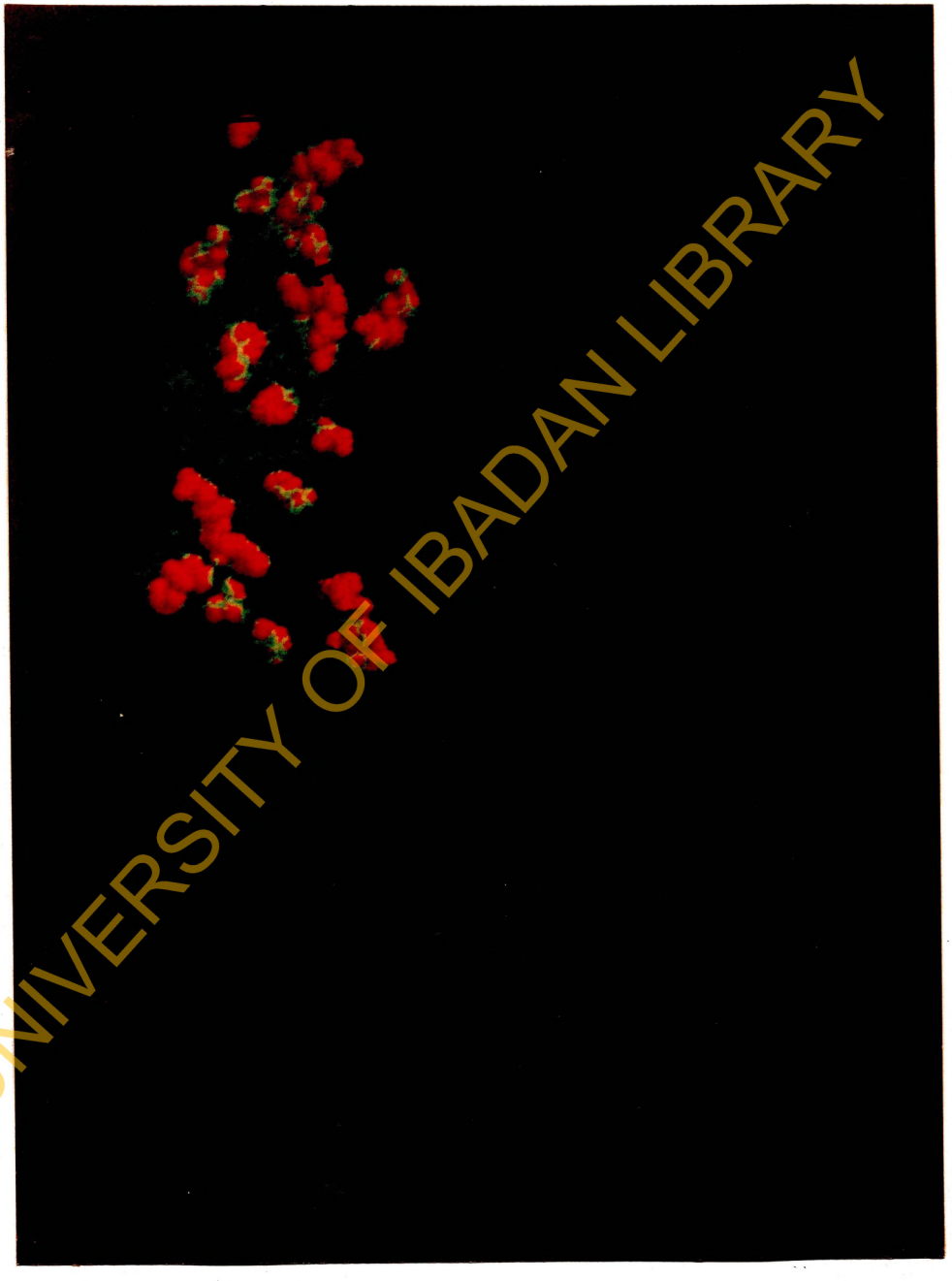


FIG:22: Phylogenetic analysis of Ibadan and other equine H3 HA genes of viruses isolated between 1963 and 1993 showing the genetic relationship and evolution of viruses. The horizontal distance is proportional to the maximum number of nucleotide differences needed to join the gene sequence (no scale is used) while the vertical lines are for spacing branches and lables.

Plate 9: A monomer of H3 HA molecule showing the amino acid changes seen in the 1989/93 cluster viruses in Yellow while red shows changes unique to Ibadan viruses. HA1 is light blue and HA2 is dark blue.



4.4 DISCUSSION

In this chapter the three Ibadan virus isolates were characterised at the molecular level to determine their origin, genetic relatedness and evolution. The reverse transcription results confirm the Ibadan viruses' RNAs as influenza A virus RNAs. The absence of subgenomic/defective interfering (sg/DI) RNAs in these new virus isolates as seen in their RNA transcripts is probably a reflection of their passage history (see materials and methods in chapter 2) which gave a low to moderate multilplicity of infection (m.o.i.) in contrast to laboratory adapted viruses with long passage history. When influenza virus is serially passaged at high m.o.i. of >10, small RNA species called sg/DI RNAs derived predominantly from the polymerase genes (PB1, PB2 and PA) may become detectable (Nayak *et al.*, 1985). At least three or four consecutive undiluted passages of virus are required to yield substantial amounts of DI particles even when sg/DI RNAs are already present in the virus stock (Janda *et al.*, 1979). Since the present virus isolates were diluted and passaged only three or four times to obtain the working stocks, it is very unlikely that infection of monolayer cells with these viruses before i.c. RNA extraction would have been at a m.o.i. >10. Two model mechanisms, the 'jumping polymerase' (Jennings *et al.*, 1983) and the 'rolling polymerase' (Nayak *et al.*, 1985) have been proposed for generating DI or suppression of genomic RNA replication. It is unlikely that either mechanism occurred in the RT reaction above since neither the RNA replication was suppressed nor sg/DI RNAs were generated.

The nucleotide sequences of the genes encoding the polypeptides of the ribonucleoprotein (RNP) complex and the non-structural polypeptides show that all the genes are most closely related to those of equine H3N8 viruses of the western hemisphere. This result is in contrast to that of an H3N8 virus isolated from horses in Northern China in 1989 (A/Eq/Jilin/1/89) which was a virus of an avian origin. The partial nucleotide sequences of the Ibadan viruses showed variation from the sequences of other examples of equine influenza viruses available in the nucleotide database and these are shown in tables 12-14. In all cases except RNA segment 3, when sequence data were available in the database, the closest relationship of nucleotide sequence was to viruses isolated in the USA in 1986, but it must be noted that these sequences deposited in the data bases are those of the most recent virus isolates. For RNA segment 3, a 1973 equine H7N7 virus gene from A/Eq/London/1416/73 was the closest relative which re-echoes the exchange of H7N7 equine influenza virus internal genes of viruses isolated after 1972 with H3N8 virus internal genes (Adeyefa *et al.*, 1994). But whether this relationship would be reflected over the whole gene is a point which has not been addressed since only a limited sequence analysis was carried out. Overall, an insufficient number of examples of equine influenza virus gene sequences are available in the databases to enable conclusions of potential epidemiological significance relating to the evolutionary origins of the genes of the RNP complex and the non-structural genes of these Ibadan viruses to be made other than that they are of equine-2 influenza virus origin rather having an avian or other source.

The complete nucleotide sequences of the HA genes of the three Ibadan isolates have been determined and phylogenetic analysis shows that the Ibadan viruses are most closely

related to viruses isolated contemporarily from Northern Europe and the Far East in Hong Kong and North and South America (Fig. 22). The Northern European viruses appear likely to be the result of a process of antigenic drift from the earlier strains of the 1980s (Berg *et al.*, Binns *et al.*, 1993; Oxburgh *et al.*, 1993, 1994). This proposed antigenic drift as opposed to genetic drift or shift is manifest by an increase in the number of amino acid changes that become fixed and which map to the antigenic sites of the HA molecule. Two of the Ibadan viruses (Ibadan/4/91 and Ibadan/6/91) showed some variations from the best characterised Northern European strain of virus (A/Eq/Suffolk/89) and these base changes resulted in amino acid changes which are unique to these Ibadan viruses particularly A/Eq/Ibadan/4/91 and A/Eq/Ibadan/6/91 and which also map to antigenic sites or were within the signal sequence in the HA1 domain and are also a result of antigenic drift. Similarly the HA gene sequence of a virus (A/Eq/Hong Kong/92) isolated from horses in Hong Kong in a recent wave of equine influenza outbreaks around the world including Argentina inspite of recent vaccination and stringent control strategies has been shown to be closely related to the European prototypic virus (Suffolk/89) with eleven nucleotide substitutions of which four resulted in amino acid changes (Lai *et al.*, 1994) also as a result of antigenic drift. A/Ibadan/9/91 belonged to another phylogenetic lineage containing isolates from Europe (Arundel/12369/91 in UK) and elsewhere (LaPlata/1/93 in South America). Only a single amino acid change (aspergine to serine at residue 290) was seen (in antigenic site C) when the sequence of the HA1-encoding protein of the HA gene was compared to Arundel/12369/91 but in other examples of viruses in the same phylogenetic lineage, this am acid substitution was not conserved and was like that in Ibadan/9/91

(Chambers *et al.* 1994). This is very significant and all these results taken together with those of the Ibadan viruses' HA gene sequences and of other viruses isolated from 1983-1993, it now appears that the HA genes of recent equine-2 virus isolates are evolving at about 50% faster rate since the mid-1980s than previously (see Fig. 8 in chapter 1 and Fig. 22 in this chapter, Lai *et al.* 1994, Webster and Kawaoka 1994). The change at residue 135 in two of the viruses (Ib4 and Ib6) introduces a new glycosylation site at residue 133 nearby (NGR to NGT) and this glycosylation site is not present in other recently isolated equine influenza viruses. There is no direct evidence for its use (besides the increased infectivity of these viruses observed in chapter three above (see page 133) but the mobility of the HA in SDS gels appears distinct from that of a virus (A/Eq/Ibadan/9/91) that does not have this site (Plate 3). The utilization of a new glycosylation site may be significant antigenically since it is analagous to the observation by Skehel *et al.* (1984) of the acquisition by an epidemic strain of human influenza virus (A/England/878/69) of a new glycosylation site at position 63 and was a variant selected by a monoclonal antibody which affected the antigenicity. The ratio of 14 amino acid substitutions in total in the three viruses (10 of which are in the antigenic sites) to 16 silent coding-changes cannot be taken as direct proof of antigenic drift having occured driven by natural selection. Moreover, the amino acid changes which map outside the antigenic sites are unlikely to have been selected directly by antigenic selection but they may have been selected as a result of genetic linkage of antigenic drift by association with the other mutations and the low ratio of non-synonymous-to-synonymous mutations may be caused by strong constraints to variation within the regions of HA glycoprotein that are not exposed in the three

dimensional structure of the molecule.

Some variation in the HA nucleotide sequence was seen among the three Ibadan viruses studied here in great detail. The sites of variation in amino acid sequences in the HA1 domain map to antigenic sites of the HA molecule. A possible cause of variation within closely related virus isolates is that of selection of antigenic variants imposed during isolation, usually in embryonated hen eggs (recently reviewed by Robertson, 1993). The possibility of host-cell selection has been recently examined in equine influenza viruses (Ilobi *et al.*, 1994) but selection appeared to be imposed by passage of virus in tissue culture. The amino acid sequence variation seen among the three Ibadan viruses isolated in embryonated hen eggs were at positions 6, 135 and 244 of HA1 and position 43 of HA2. These sites have not been described as sites that are subject to host cell selection in human H3 viruses (reviewed by Robertson, 1993). Likewise, the single amino acid difference observed between Ibadan/9/91 and Arundel/12369/91 at position 290 has not been a residue for host cell selection in human H3 viruses. However, the differences between the amino acid sequences of Ib4 and Ib6 on the one hand and those of Ib9 on the other hand at positions 138 and 156 of HA1 have been seen to be associted with egg adaptation of human virus strains and may similarly reflect *ex-vivo* selection in these Ibadan viruses. Two alternative explanations for the micro-heterogeneity among the three virus strains could be simple genetic drift or antigenic drift and the likehood that they reflect the differences in the original viruses present in the affected animals. However, the possibility that host cell selection has resulted in the heterogeneity among the three viruses cannot be

discounted. The fact that there were base changes among the three viruses which resulted in amino acid variation in recognised antigenic sites of the HA could be presented as an argument for antigenic selection, albeit antigenic drift is manifest at virus population rather than at individual isolate level.

Comparision of amino acid sequences at the cleavage site of the HAs of the Ibadan viruses revealed the conservation among the HAs of these viruses and all HAs examined to date, of two amino acids: the proline proximal to the C-terminus of the HA1 and the terminal glycine of the HA2. Also revealed was the conserved arginine residue at the C-terminus of HA1, the cleavage site, which is conserved in all but the H14 strains of influenza A viruses (Kawaoka *et al.*, 1990; Nobusawa *et al.*, 1991). The conserved proline upstream of the cleavage site may be very important in presentation of the cleavage site to an intracellular protease owing to its strong turn-breaking potential. It has been reported (Walker and Kawaoka, 1993) that the arginine residue at the C-terminus of HA1 is absolutely required for HA cleavage by cellular proteases and that at least five or six basic amino acid residues are required for maximal cleavage by intracellular proteases. The arginine residue at the C-terminus of HA1 is also very important for abolition of steric hinderance near the cleavage site. The presence of these three amino acids at the cleavage site of the present virus isolates suggests that equine H3 HAs are cleaved *in-vivo* by intracellular proteases which are abundant in the respiratory tracts of equines in natural infection thus rendering the viruses infectious although the HAs of equine H3 viruses are not cleaved in tissue culture in contrast to equine H7 virus HAs as shown in chapter 2.

In conclusion, this study describes the detailed molecular characterisation of the first equine influenza viruses isolated from equines in tropical Africa. The results show that two of the three independently isolated viruses are more closely related to each other than to the third one and although they are antigenically divergent they exhibit the structural and biological as well as antigenic and genetic characteristics of equine-2 (H3N8) influenza viruses. From comparision with other influenza A virus genes, the inference that could be drawn is that equine viruses isolated in tropical Africa are more closely related to equine viruses from Europe in one case (Ib4 and Ib6) and in the other, from Europe and elsewhere (Ib9) rather than to an earlier isolate from temperate South Africa or North Africa with two distinct viruses being present simultaneously in Nigeria. Equine influenza viruses isolated in Hong Kong in 1992 were epizootiologically related to contemporary viruses from Europe (Lai *et al.* 1994) whereas the epizootiology of those from mainland China isolated during 1993/94 is less clear (Shortridge *et al.* 1995, Guo *et al.* 1995). Although in Nigeria there are fewer opportunities for the introduction of viruses from abroad than in Hong Kong which has numerous links with the bloodstock industry of the Middle East and Western Hemisphere, the posssible source of these viruses would be introduction from imported horses in view of the recent increase in transcontinental movement of horses and the recent worldwide outbreaks of equine-2 influenza in North and South America, Europe and Scandinavia, Indian Sub-continent and China, Japan and Hong Kong in the Far East as cited in chapter 1 above. The nucleotide and amino acid changes observed in the present virus isolates and other recent isolates indicate antigenic drift among equine H3N8 influenza viruses as earlier reported (Kawaoka *et al.*, 1989a) and confirm that drift strains

of equine-2 viruses can co-circulate in equine populations. A few of these amino acid changes are located in the conserved or functional domain of the HA1 such as the antigenic sites or within the signal sequence. The results of these studies and the observations made confirm the occurence and existence of equine influenza viruses in Nigeria in a tropical environment and the simultaneous isolation of viruses of two phylogenetically distinct lineages early in 1991 in Nigeria reinforces the importance of constant global monitoring of equine influenza viruses, it again demonstrates and re-emphasise the rapid global spread of influenza viruses around the world and highlights the risks that accompany transportation and crowding of horses at tournament, race meetings or markets.

The TA Cloning™ system used in this study provides a quick one-step cloning strategy, taking advantage of the non-template dependent activity of the thermostable DNA polymerase used in PCR amplification that adds deoxyadenosines to the 3' end of all duplex molecules. The A-overhangs are used to insert the cDNA (PCR products) into the TA cloning vector that provides single T-overhangs at the insertion site. The method of multiplex RT/PCR and cycle sequencing developed for rapid analysis of influenza virus genes and employed in the present study has great potentiality in viral molecular epidemiology, rapid diagnosis and virus surveillance. Although the error frequency of Taq polymerase used is approximately 2×10^{-4} per base duplication (Keohavong and Thilly, 1989), direct sequencing of the dominant population of PCR products using segment specific end-labelled oligonucleotides reduces misincorporation errors by Taq DNA polymerase on the sequence ladder as demonstrated in this study and a previous one (Adeyefa *et al.*, 1994). For direct detection and amplification of viral RNA/cDNA in

clinical specimens, the access to virus genes without adaptation in cultured cells or chicken embryo allantoic and amniotic membrane cells avoids mutational selection which may occur during passaging of the virus. Although attempts to detect the virus RNAs/cDNAs directly from the clinical nasopharyngeal swabs obtained from equines gave variable results which were sometimes not quite satisfactory and this technique was therefore not used in this study, studies are still in progress to fully develop and optimise this technique since it has a greater potential of rapid diagnosis of influenza infection in the different host species.

CHAPTER 5

CONCLUSIONS

The result of this work confirm that equine influenza virus occurs in Nigeria and that at least the equine-2 H3N8 subtype have previously circulated and may be circulating among the equine populations in this country based on the isolation of these equine-2 viruses during a recent nationwide surveillance over a 5 year period. The three viruses that were isolated were respectively named A/Equine/Ibadan/4/91, A/Equine/Ibadan/6/91 and A/Equine/Ibadan/9/91.

Although viruses were not isolated from samples collected subsequent to December 1991, this could be due to the immunity in equines in the country due to previous exposure to these viruses as antibodies to equine-2 influenza viruses have been demonstrated in the sera of horses collected from 1989 to 1993 (Adeyefa *et al.*, 1995). It is however not unusual for the disappearance of a circulating virus followed by re-emergence of novel viruses after a period of time due to antigenic drift or genetic drift. Their broad reactivity in antigenic cross-reactivity with panels of monoclonal antibodies (Mabs) and polyclonal antisera suggests that the viruses are similar to but not identical with equine-2 influenza viruses isolated in Europe and USA between 1963 and 1987. Antigenic analysis indicated that the three viruses were antigenically divergent and the proteins of the ribonucleoprotein complex were observed to be heterogenous among the three viruses. The conclusions that could be safely reached were that the genes of these viruses, though equine in origin, were related to some other subtype influenza A viruses and that the number of nucleotide substitutions in their HA genes particularly those that resulted in amino acid

changes were responsible for their antigenic diversity. The HA glycoproteins of the viruses were not cleaved in tissue culture in contrast to those of H7 equine and H7 and H5 pathogenic avian viruses, probably due to the absence, at the cleavage site of the H3 HAs, of a series of basic amino acids and the nine amino acid insertion observed in the H7 equine viruses. This non-cleavability could serve to distinguish the equine, human H3 HAs and the non-pathogenic avian HAs from those of others that are cleaved in tissue culture particularly when there is low and inconclusive reactivity with antisera and Mabs.

The viruses were sufficiently infectious to cause infection which induced protective neutralizing antibodies in an equine model. Their glycoproteins, both intact and denatured, were antigenic and immunogenic. Based on the results of antigenic and immunogenicity studies and the present understanding of the immunological response of equines when exposed to influenza virus antigens, it is recommended that the virus glycoproteins could be used as immunogens for the protection of valuable animals.

From genetic analysis, the virus genes are equine in origin and despite the unusual area of isolation of the viruses, their genes are very closely related to those of equine viruses contemporarily causing disease in the Western Hemisphere and they are from the same evolutionary tree. The nucleotide and amino acid changes in the viruses' HA genes particularly the changes unique to these Ibadan viruses either mapped to antigenic sites or were within signal sequence in the HA1 domain. These changes resulted from a process of considerable antigenic drift away from the original equine-2 prototype viruses and were responsible for the antigenic diversity among the three viruses. Phylogenetic analysis indicates that recent equine-2 H3 HAs are evolving at a faster rate of about 50% than they

were in the late 1960s and mid-1970s. The results of molecular analysis confirm that drift strains of equine-2 influenza viruses could and did circulate among equine populations in Nigeria. The possible source of these viruses would be introduction of imported horses into a susceptible population. These results thus serves to re-emphasize the potential of influenza viruses for a rapid global spread and hence the necessity for constant virus surveillance around the world as well as for effective control measures through annual vaccination using adequately upgraded vaccines and strict quarantine of newly introduced animals.

This work describes the detailed molecular characterisation, antigenicity and immunogenicity of the first equine influenza viruses isolated in tropical Africa, albeit the third from the African continent. It has also provided the hitherto unavailable information on the status of equine influenza in this part of the world. These viruses exhibited the structural and biological as well as antigenic and genetic characteristics of equine-2 (H3N8) subtype of influenza A viruses. The molecular study was achieved using the technique of multiplex RT/PCR and cycle sequencing developed for rapid analysis of influenza viruses genes. The results of this and other work (Adeyefa *et al.*, 1994) demonstrate the potential of this technique for wider application in molecular epidemiology, rapid diagnosis and surveillance studies not only of influenza viruses but some other human and animal viruses as well. The method of non-radioactive lympho-proliferation assays also facilitated the immunological aspect of this work.

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APPENDIX A: MEDIA

Growth medium

(a) Dulbecco's Modified Eagle's Medium (DME): 25mM Hepes, Earl's salt, L-glutamine, 10% fetal calfs serum, 1000mg glucose/L, pyridoxine hydrochloride, 0.01% penicilin, 0.01% streptomycin, 0.00025% fungizole, 0.06% sodium bicarbonate, sterile distilled water.

(b) GIBCO Medium 199: 25mM Hepes, Earle's salt, L-glutamine, 10% fetal calf serum, 0.01% penicilin, 0.00025% fungizone, 0.06% sodium bicarbonate, sterile distilled water.

(c) Minimum Essential Medium: Earle's salt, 0.6% sodium bicarbonate, L.glutamine, 200μg/ml penicilin, 100μg/ml streptomycin, sterile distilled water.

Maintenance medium

(a) Medium 199, 2% calf serum, 0.01% streptomycin, 0.01% penicilin, 0.00025% fungizone, 0.06% sodium bicarbonate, sterile distilled water.

(b) Eagle's medium: 6.4gm NaCl, 0.48gm KCl, 0.2gm, $MgSO_4$, $7H_20$, 0.1% Fe_3N $9H_20$, 0.148m Na H_2 $P0_4$, 0.292gm glutamine 0.28gm $CaCl_2$, 2.75gm $NaHC0_3$, 15μl phenol red, 50μl stock amino acids, 4ml vitamin concentration, 0.01% penicilin, 0.01% streptomycin, 0.00025% fungizone per liter.

(c) Eagle's medium with 2% adult bovine serum

SOC

SOC medium: 2% Bacto tryptone, 0.5% Bacto yeast extract, 10mM sodium chloride, 2.5mM potassium chloride, 10mM magnessium chloride, 10mM magnessium sulphate, 20mM glucose.

Luria-Bertani medium: 1% Bacto tryptone w/v, 0.5% Bacto (LB broth)yeast extract w/v, 0.1% sodium chloride w/v adjusted to pH 7.5 with sodium hydroxide.

LB plates — LB broth plus 1.5% agar

APPENDIX B: Buffers and Solutions

10X TBE: 0.89M Tris-base pH8.3, 0.89M Boric acid, 0.02M EDTA

1X TBE: 100ml 10X TBE, 900ml dionised water

5X Reverse transcript buffer: 350mM KCl, 250mM Tris pH 8.2, 40mM $MgCl_2$ 50mM DTT

5X TSP buffer: 250mM Tris-HCl pH9.5, 7.5mM $MgCl_2$, 100mM $(NH_4)_2SO_4$

10X Taq buffer: 100mM Tris-HCl, 15mM $MgCl_2$, 500mM KCl, 1mg/ml gelatine

10X T4 polynucleotide kinase buffer: 500mM Tris-HCl pH 7.5 at 25°C, 100mM $MGCl_2$, 50mM DTT, 1mM Spermidine

5X fmol Taq sequencing buffer: 250mM Tris-HCl pH 9.0 at 25oC, 10mM $MGCl_2$

10X Circumvent sequencing buffer: 10mM KCl, 100mM $(NH_4)_2SO_4$, 200mM Tris-HCl pH 8.8, 50mM MgS04

fmol sequencing stop solution (Promega): 10mM NaOH, 95% formamide, 0.05% bromophenol, 0.05% xylene cyanol

Circumvent sequencing stop/loading solution (New England Biolabs): 95% Deionised formamide, 0.3% xylene cyanol FF, 0.3% bromophenol blue, 0.37% EDTA pH 7.0

Formamide dye: 10mM Tris-HCl pH 7.4, 2.5mM EDTA pH 8.0, 0.05% xylene cyanol, 0.05% bromophenolblue, 95% formamide

30X Triton X-100: 3% Triton X-100

Glycerol dye:
(a) 84% glycerol, 50mM Tris-HCL, 10mM EDTA, 0.05% bromophenol blue

(b) 50% glycerol, 1X MOPs, 0.05% bromophenol blue.

Sucrose dye: 6X TBE, 0.25% bromophenol blue, 40% w/v sucrose in water

Tris EDTA (TE): 10mM Tris HCL pH 8.0, 1mM EDTA

Ligase buffer: 50mM Tris HCL pH 8.0, 8mM $MGCl_2$, 5mM DTT

Transformation buffer: 100mM KCl, 45mM $MGCl_2$, 10mM $CaCl_2$, 3mM hexamine cobalt chloride, 10mM K-Mes pH 6.3

Protein gel sample buffer: 8M Urea, 2% SDS, 2% 2-mercaptoethanol, pH 6.8, 10mM Tris -HCl pH 7.4, 0.05% bromophenol blue

Lyse buffer: 50mM sodium acetate pH 5.0, 2.5mM EDTA pH 8.0, 0.5% SDS

Mini-prep solution I: 50mM glucoe, 25mM Tris-HCl pH 8.0, 10mM EDTA pH 8.0

solution II: 1% SDS, 0.2M sodium hydroxide

solution III: 3M potassium acetate pH 4.8, (60ml 5M potassium acetate), 11.5ml glacial acetic acid, 28.5ml sterile double distilled water.

Sucrose solutions:

60% 60gm succrose in 100ml PBS

45% 45gm succrose in 100ml PBS

30% 30gm succrose in 100ml PBS

15% 15gm succrose in 100ml PBS

Tris-saline: 5ml Tris-HCl pH 8.0, 500ml saline

Ethidium bromide: 100mg in 10ml MQ

0.6% Thiobarbituric acid: 0.6gm thiobarbituric acid in 100ml distilled water

Sodium meta-periodate solution: 4.28gm sodium-M periodate (Sigma) in 100ml distilled water plus 62ml concentrated orthophosphoric acid.

50% Arsenite reagent: 25gm sodium arsenite (Sigma) in 50ml distilled water plus 0.75ml concentrated sulphuric acid.

Fetuin solution: 100mg fetuin (Sigma) dissolved in 2ml sterile distilled water plus 2ml 0.4M PBS pH 5.9.

Sialic acid (standard 1mg/ml): 2mg N-acetyl neuraminic acid (NANA-Sigma) in 2ml sterile distilled water.

10% Sarkosyl: 50ml sarkosyl in 500ml distilled water

1M Tris-HCl pH 8.0

2M sodium acetate pH 4.0

0.4M Tris-HCl, 0.2M sodium acetate pH 9.8

3M sodium acetate pH 5.5

0.5M sodium acetate pH 5.0

1M sodium acetate pH 5.5

1M Tris.HCl pH 9.2

10M ammonium acetate

1M Tris-HCl pH 8.4

3M sodium acetate pH 5.2

0.5M sodium acetate/Tris-HCl pH 5.0

1M Tris-HCl pH 8.2

1M Tris-HCl pH 6.8

0.1M potassium periodate

10M sodium hydroxide

1M sodium hyc oxide

1M sulphuric acid

1M Tris-HCl pH 7.4

5M sodium chloride

80% glycerol

Isopropanol

Ethanol

Chloroform

10% SDS

Butanol (water saturated)

Butan-1-ol

3M potassium chloride

1.5M Tris-HCl pH 8.7

1M Tris-HCl pH 7.8

3M potassium acetate

0.1M Tris-HCl pH 7.4

0.1M sodium acetate pH 5.0

1M Tris-HCL pH 9.4

2M Tris-HCl pH 8.0

10M Urea

0.5M EDTA pH 8.0

0.1M calcium chloride

1M ammonium sulphate

1M sodium chloride

50% polyethylene glycol

70% ethanol

1M magnessium sulphate (filtered)

0.4M PBS pH 5.9

Diethyl ether

Water and TE saturated phenol

Methylated spirit

3% Tegadoor

B-mercapto-ethanol

Guanidine thiocyanate

Methanol

Glacial acetic acid

Xylene cyanol

Bromophenol bleu

TEMED

10mM methionine

Repelcote

Decon

Lipsol

Fam Concentrated ammonia

Iso-amyl alcohol

APPENDIX C: EQUIPMENT AND LABORATORY MATERIALS

Biometra Trio-Thermoblock TB1

PTC-100 Programmable Thermal Controller (MJ Research Incorporated)

G24 Environmental Incubator Shaker (New Brunswick Sci. Instrument)

Egg punch gun serial number 2912

-70°C Revco freezer, -20°C Freezer, 4°C refrigerator

Costa 25, 50, 100 and 200 μl Octa pipete

MSE FISONS Centrifuge

Beckman Ultracentrifuge models L5-50, L8-55, L8-70, L8-55M (Beckman Instrument Incorporated)

Sucrose gradient vacuum pump

Ultraclear centrifuge tubes

RC5B Sorval Refrigerated Superspeed centrifuge

Eppendorf microfuge 5414

Ultrasonic sonicator model W-375 (Heat Systems Ultrasonics, Incorp)

Thomson microwave oven

Whatman laboratory sealing film

Sarsdect and Eppendorf microfuge tubes

Denley metal cupboards

Whirlemixer

SpeedVac (Uniscience Univap plus Savant refrigerated condenser trap)

Shimadzu UV-150-02 double beam spectrophotometer

Bromma LKB power pack model 2197

Falcon 50ml centrifuge tubes

IEC Centra 4R centrifuge

Grant CO_2 incubator

Radman radioactive monitor model 5-10EL (Mini Instrument Ltd.)

Whatman 3mm CHR 19cm filter paper

Vacuum slabgel dryer Sr. model SE 1160 (AERG)

Kodak X-OMAT M20 Processor

Polaroid 4 MP-4 Land Camera (Photodyne Incorporated)

10, 20, 200 and 1000μl Gilson's micro pipete and tips

Polaroid Type 667 coaterless black and while Land films (Polaroid Corporation, Cambridge, Massachusset)

Eppendorf micropipet and gel loading (Stratstip) tips

Minigel Sumbarine Gel system (Gallenkamp)

Microfuge tube racks

Phillips UV Scan spectrophotometer

X-ray film illuminator

Mini-gel apparatus (ScotLab)

Acrylamide gel apparatus

Hybaid Intelligent Heating Block model IHB 2024

Denley bench top general purpose centrifuge

Laminal flow sterile hood

Autoradiography X-ray films (Genetic Research Instruments, Fuji, Kodak)

20 x 20, 40 x 20 and 50 x 20 glass gel plates

Nicon microscope and camera mounting

Swab sticks

other Laboratory Consumables.

APPENDIX D: GEL MIXES

1% agarose	1gm agarose in 100ml 1X TBE
4% polyacrylamide	8M urea, 1X TBE, 4% acrylamide, 0.2% bisacrylamide, 0.1% w/v ammonium persulphate, 0.1% TEMED
6% polyacrylamide	8.3M urea, 1X TBE, 6% acrylamide, 0.3% (sequencing) gel bisacrylamide, 0.1% w/v ammonium persulphate, 0.1% TEMED
20% polyacrylamide	8.3M urea, 1X TBE, 20% acrylamide, 0.5% bisacrylamide, 0.1% w/v gel ammonium persulphate, 0.1% TEMED
12.5% SDS polyacrylamide	12.5% acrylamide, 1% bisacrylamide, 375mM (running) protein gel Tris. HCl pH 8.7, 0.1% SDS, 0.1% ammonium persulphate, 0.1% TEMED
15% SDS polyacrylamide	15% acrylamide, 0.7% bisacrylamide, 375mM (running) protein gel Tris. HCl pH 8.7, 0.1% SDS, 0.1% ammonium persulphate, 0.1% TEMED
5% stacking protein gel	5% acrylamide, 0.13% bisacrylamide, 125mM Tris.HCl pH 6.8, 0.1% SDS, 0.1% w/v APS, 0.1% TEMED
	Sephadex G50 (fine)

APPENDIX E: ENZYMES

(1) Avian myeloblastosis virus (AMV) Reverse Transcriptase (Promega) 8 units/μl

(2) RNAsin (RNAse inhibitor) (Stratagene) 40 units/μl

(3) Type II Thermophilic DNA polymerase (TSP-Cambio, Cambridge, England) 5 units/μl

(4) Taq DNA polymerase (Boerhinger Mannheim) 5 units/μl

(5) Taq DNA polymerase sequencing grade (Promega) 5 units/μl

(6) T4 polynucleotide kinase (Epicenter) 40 units/μl

(7) T4 polynucleotide kinase (Promega) 8 units/μl

(8) Vent^{TM}R (exo^{-}) DNA polymerase (New England Biolabs) 2 units/μl

(9) Eco Rl (Promega) 5 units/μl

(10) RNAse A

(11) T4 DNA ligase (Invitrogen)

APPENDIX F: Oligonucleotide primers

1st strand primer (Rostock 1-12, 3' end)	
AGCAAAAGCAGG	(12 Mer) A 6, C2, G4
2nd strand p. mer (Rostock 1-13 5' end)	
AGTAGAAACAAGG	(13 Mer) A7, C1, G4, T1
Oligothymidylic acid (Oligo dT, d(p)T 12-18 Sigma	
GTCAAAT	(7 Mer) A3, G1, C1, T2
Segment 1:3'	
AGCAAAAGCAGGTCAAAT	(18 Mer) A9, C3, G4, T2
Segment 2:3	
AGCAAAAGCAGGCAAACT	(18 Mer) A9, C4, G4, T1
Segment 3:8-27	
GCAGGTACTGATCCAAAATG	(20 Mer) A7, C4, G5, T4
Segment 5 NP 1-24	
AGCAAAAGCAGGGTAGATAATCAC	(24 Mer) A11, C4, G6, T3
Segment 6 N8:7-26	
AGTTTAAAATGAATCCAAAT	(20 Mer) A10, C2, G2, T6

Segment 7:1-25

AGCGAAAGCAGGTAGATATTGAAAG (25 Mer) A11, C2, G8, T4

Segment 8 V8:8-31

GCAGGGTGACAAAAACAT ATGGA (24 Mer) A11, C3, G7, T3

H3Eq/HA1 : 8-27

GCAGGGGATATTTCTGTCAA (20 Mer) A5, C3, G6, T6

H3Eq/HA 101-124

CACAGCCACATTATGTCTGGGAGA (29 Mer) A7, C7, G5, T5

H3Eq/HA2: 151-170

TGGTAAAAACAATAACTGAT (20 Mer) A10, C2, G3, T5

H3Eq/HA3: 315-333

CAGTATGAGAATTGGGACC (19 Mer) A6, C3, G6, T4

H3Eq/HA4: 481-498

GAAGTGGGGCCTGCAGAA (18 Mer) A5, C4, G7, T2

H3Eq/HA5: 661-680

ATGTCCAACAATTAGGGGGA (20 Mer) A7, C3, G6, T4

H3Eq/HA6: 841-859

GGGGATATTTCAAAATGCG (19 Mer) A6, C2, G6, T5

H3Eq/HA7:

ACAAATGTGATAATGCATG (19 Mer) A8, C2, G4, T5

H3Eq/HA8

GTAACAGTCTCAAGAAAG (18 Mer) A8, C3, G4, T3

H3Eq/HA9 Reverse

CCGACCCTTCCTTACCAACTA (21 Mer) A5, C10, G1, T5

H3Eq/HA200 Reverse

3'OH GTTTACGATGACTTAAT 5'P (17 MeR) A5, C2, G3, T7

H3Eq/HA256 Reverse

CCATCTAGAACCCTATATGAG (21 Mer) A7, C6, G3, T5

H3Eq/HA650

AAATTGTATATCCAAGAA (18 MeR) A9, C2, G2, T5

H3Eq/HA701

TCAACAAACAGTAATCCC (18 MeR) A8, C6, G1, T3

H3Eq/HA950 Reverse

TTTGTCGTTGGGGATGCT (18 Mer) A1, C2, G7, T8

H3Eq/HA1000 Reverse

ATATACTTGGGGCATTTTC (19 Mer) A4, C3, G4, T8

H3Eq/HA1740 Reverse

5' AACTATCAGTTTACTCAAATGCA (23 Mer) A9, C5, G2, T7

(Oligonucleotide primers from Dr. R. G. Webster in Memphis)

ET 9

CAGGGGATATTTCTGTCA (18 Mer) A4, C3, G5, T6

ET 268

CATTAATAGATGCAATGC (18 Mer) A7, C3, G3, T5

ET 444

GGATTCACATGGACAGGT (18 Mer) A5, C3, G6, T4

ET 592

ATTTCGATAAACTATACA (18 Mer) A8, C3, G1, T6

ET 714

ATCCCCAACATCGAGT (16 Mer) A5, C6, G2, T3

ET 879

AGATCAGATGCACCCATA (18 Mer) A7, C5, G3, T3

ET 1032

ATGAGGAATGTACCAGAA (18 Mer) A8, C2, G5, T3

ET 1182

ACTCAAGCAGCCATCGA (17 Mer) A6, C6, G3, T2

ET 1277

AGTAGAAGGGAGAATCC (17 Mer) A7, C2, G6, T2

ET 1368

GAAAATCAACATACAATT (18 Mer) A10, C3, G1, TA

ET 1526

GACATATGACCATTAAC (16 Mer) A6, C4, G2, T4

Oligonucleotide primers from Promega

pUC/ M13 Forward

GTTTTCCCAGTCACGAC (17 Mer) A3, C6, G3, T5

CGCCAGGGTTTTCCCAGTCACGAC (24 Mer) A4, C9, G6, T5

pUC/ M13 Reverse

CAGGAAACAGCTATGAC (17 Mer) A7, C4, G4, T2

TCACACAGGAAACAGCTATGAC (22 Mer) A9, C6, G4, T3

APPENDIX G: Composition of Nucleotide Mixes

cDNA cycle sequencing with end-labelled primers

Components	Deaza G Nucleotide Mix	Deaza A Nucleotide Mix	Deaza T Nucleotide Mix	Deaza C Nucleotide Mix
ddGTP	120µM			
ddATP		1400µM		
ddTTP			2400µM	
ddCTP				800µM
7-deaza dGTP	80µM	80µM	80µM	80µM
dATP	80µM	80µM	80µM	80µM
dTTP	80µM	80µM	80µM	80µM
dCTP	80µM	80µM	80µM	80µM

RNA non-cycle sequencing

(a) End-labelled RNA sequencing

Components	G Nucleotide Mix	A Nucleotide Mix	T NNucleotide Mix	C Nucleotide Mix
ddGTP	80µM			
ddATP		80µM		
ddTTP			80µM	
ddCTP				80µM
dGTP	400µM	400µM	400µM	400µM
dATP	400µM	400µM	400µM	400µM
dTTP	400µM	400µM	400µM	400µM
dCTP	400µM	400µM	400µM	400µM

(b) Internal-labelled (Direct incorporation) RNA sequencing

Components	G Nucleotide Mix	A Nucleotide Mix	T Nucleotide Mix	C Nucleotide Mix
ddGTP	25μM			
ddATP		8μM		
ddTTP			80μM	
ddCTP				25μM
dGTP	60μM	60μM	60μM	60μM
dATP	60μM	60μM	60μM	60μM
dTTP	60μM	60μM	60μM	60μM
dCTP	60μM	60μM	60μM	60μM

Appendix H: Radio-isotopes (NEW England Nuclear).

(1) γ^{32}P-ATP : 3μl of 3000 Ci/mmol at 10μCi/μl =30μCi= 10pmoles

(2) α^{32}P-dATP: 0.5μl of 800 Ci/mmol at 10μCi/μl

(3) α^{35}S-dATP: 0.5μl Of 1,000 Ci/mmol at 10μCi/μl

(4) ^{35}S-Methionine : 1232.7 Ci/mmol at 100μCi/μl in Earle's salt.