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Phylogenetic analysis of H7 haemagglutinin subtype influenza A viruses*

Brief Report

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Summary. A 945 nucleotide region (bases 76–1020) of the HA1 part of the HA gene was obtained for 31 influenza viruses of H7 subtype isolated primarily from Europe, Asia and Australia over the last 20 years. These were analysed phylogenetically and compared with sequences of the same region from 23 H7 subtype viruses available in Genbank. The overall results showed two geographically distinct lineages of North American and Eurasian viruses with major sublineages of Australian, historical European and equine viruses. Genetically related sublineages and clades within these major groups appeared to reflect geographical and temporal parameters rather than being defined by host avian species. Viruses of high and low virulence shared the same phylogenetic branches, supporting the theory that virulent viruses are not maintained as a separate entity in waterfowl.

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Wild aquatic birds are considered the primordial reservoir of all subtypes of avian influenza A viruses [15, 29, 30] and there is strong evidence for transmission from wild birds to domestic poultry [13]. A severe disease of domestic poultry formerly known as "fowl plague", but now termed highly pathogenic avian influenza (HPAI), is caused by certain strains of influenza virus which have the potential to spread readily to neighbouring premises. Outbreaks of HPAI are associated with the H5 and H7 subtypes of influenza A viruses and a prerequisite for pathogenicity is the presence of multiple basic amino acids at the cleavage site of the viral haemagglutinin precursor protein, rather than just two as seen in non-pathogenic influenza viruses [32]. The process by which HPAI viruses emerge is poorly understood. It is not possible to predict when introductions of HPAI to

poultry may occur, and while it seems likely that HPAI strains arise from viruses of low virulence, it is not known if the mutations leading to increased pathogenicity take place after introduction to domestic poultry or in the waterfowl reservoir, although recent theories suggest the former [11]. That HPAI viruses emerge from those of low virulence by mutation is in keeping with phylogenetic studies reported to date, as it appears that HPAI viruses within either the H5 or H7 subtype do not form unique lineages but share common ancestors with non-pathogenic H5 and H7 viruses [26]. In the present study we have examined the phylogenetic relationships between 54 H7 viruses isolated primarily in Europe, Asia and Australia over the past 20 years.

The complete coding sequence for the HA of selected H7 subtype influenza isolates held in the International Reference Laboratory repository for avian influenza at VLA-Weybridge was determined. All manipulations with infectious viruses were performed in a category III high containment laboratory. RNA extraction, reverse transcription (RT), polymerase chain reaction (PCR) and sequencing were done as described [7]. Additional published HA sequences were obtained from Genbank, all virus strains with abbreviations and accession numbers, origin or references where appropriate, are listed in Table 1. The Lasergene software package (Dnastar) was used for the assembly, analysis and translation of nucleotide sequence data. Phylogenetic analysis was performed by maximum likelihood [9], neighbor joining and maximum parsimony (results not shown) methods using the PHYLIP phylogenetic inference package (version3.57c) [10]. Some nucleotide sequences in the Genbank database are only partial; therefore 945 bases of the HA1 (76–1020) were analysed to allow the maximum number of isolates to be compared. A few North American isolates and equine strains were included to put the Eurasian and Australian isolates in context. The results are presented as an unrooted maximum likelihood phylogram, where the horizontal branch lengths are proportional to the nucleotide differences between the sequences (Fig. 1).

Minor differences were observed between the phylograms generated by the different algorithms, with the greatest agreement being between maximum likelihood and maximum parsimony. However, when bootstrapping values for the branch points of the maximum parsimony and neighbor joining trees, and probability values for maximum likelihood were considered, most of the discrepancies were at nodes of low predicted accuracy (a bootstrapping value less than 70%).

In the phylogenetic analysis (Fig. 1) the overall pattern obtained was of two geographically distinct lineages of North American and Eurasian viruses with major sublineages of Australian, historical European and equine viruses. Divergence for all the major branches corresponds loosely with the date of isolation of the viruses.

Six Australian isolates were examined, those obtained from the HPAI outbreaks in chickens in 1976 and 1985 were of H7N7 subtype, while the viruses from the more recent 1992 and 1995 HPAI outbreaks were of H7N3 subtype; the duck 1976 H7N7 isolate was not virulent for chickens [31]. These Australian viruses form a genetic lineage which is quite distinct from the Eurasian isolates and consists of four distinguishable chronological groups, 1976, 1985/86, 1992

Table 1. Viruses used for phylogenetic analysis

Virus strain	Subtype	Abbreviation	Pathogenicity[a]	Genbank accession no.
A/FPV/Weybridge/27	H7N7	FPVWe27	High	L37794
A/FPV/Rostock/34	H7N1	FPVRo34	High	M24457
A/FPV/Egypt/45	H7N1	FPVEG45	High	**AF202237**[d]
A/equine/Prague/1/56	H7N7	eqPr56	Low	X62552
A/turkey/England/63	H7N3	tyEn63	High	**AF202238**[c]
A/turkey/Oregon/71	H7N3	tyOr71	Low	M31689
A/parrot/Northern Ireland/VF-73-67/73	H7N1	paNI73	Low	**AF202252**
A/equine/London/1416/73	H7N7	eqLo73	Low	X62560
A/chicken/Victoria/76	H7N7	ckVi76	High	Z47199
A/duck/Victoria/76	H7N7	dkVi76	Low	U20463
A/turkey/England/647/77	H7N7	tyEn77	Low	**AF202247**
A/duck/Hong Kong/293/78	H7N2	dkHK78	Low	U20461
A/chicken/Leipzig/79	H7N7	ckLe79	High	U20459
A/goose/Leipzig/137/79	H7N7	goLe79	High	L43913
A/starling/England-Q/983/79	H7N1	stEn79	Low	**AF202232**
A/tern/Potsdam/342/6/79	H7N7	tePo79	Low	U20470
A/turkey/England/192-328/79	H7N3	tyEn79	Low	**AF202245**
A/turkey/Israel/Ramon/79	H7N2	tyIL79	Low	**AF202235**
A/macaw/England/626/80	H7N7	maEn80	Low	**AF202250**
A/seal/Massachusetts/1/80	H7N7	seMa80	Low	K00429
A/turkey/Minnesota/1237/80	H7N3	tyMn80	Low	U20466
A/swan/Potsdam/63/6/81	H7N7	snPo81	Low	U20467
A/chicken/England/71/82	H7N1	ckEn82	Low	**AF202236**[c]
A/chicken/Victoria/1/85	H7N7	ckVi85	High	M17735
A/starling/Victoria/1/85	H7N7	stVi85	High	M17736
A/duck/Heinersdorf/S495/6/86	H7N7	dkHe86	Low	U20465
A/chicken/Jena/1816/87	H7N7	ckJe87	Low	U20469
A/non-psittacine/England-Q/1985/89	H7N7	npEn89	Low	**AF202240**
A/chicken/Ireland/1733/89	H7N7	ckIE89	Low	**AF202239**
A/psittacine/Italy/1/91	H7N2	psIT91	Low	**AF202242**
A/ostrich/South Africa/1609/91	H7N1	osZA91	Low	**AF202244**
A/ostrich/South Africa/5352/92	H7N1	osZA92	Low	U20458
A/softbill/South Africa/142/92	H7N4	sbZA92	Low	U20464
A/chicken/Victoria/1/92	H7N3	ckVi92	High	**AF202227**
A/gull/Italy/692-2/93	H7N2	guIT93	Low	**AF202248**
A/rhea/North Carolina/39482/93	H7N1	rhNC93	Low	U20468
A/conure/England/766/94	H7N1	coEn766/94	Low	**AF202249**
A/conure/England/1234/94	H7N1	coEn1234/94	Low	**AF202241**
A/parakeet/Netherlands/267497/94	H7N1	pkNL94	Low	**AF202251**
A/parrot/England/1174/94	H7N1	paEn94	Low	**AF202243**
A/fairy bluebird/Singapore/F92/94	H7N1	fbSG94	Low	**AF202229**
A/chicken/Pakistan/447/95	H7N3	ckPK447/95	High	**AF202226**
A/chicken/Pakistan/CR2/95	H7N3	ckPKCR2/95	High	**AF202230**
A/chicken/Pakistan/16/99/95	H7N3	ckPK16/95	High	**AF202233**
A/chicken/Queensland/667/95	H7N3	ckQu95	High	**AF202231**

Table 1 (*continued*)

Virus Strain	Subtype	Abbreviation	Pathogenicity[a]	Genbank accession no.
A/common iora /Singapore/F89/95	H7N1	ciSG95	Low	**AF202228**
A/turkey/Ireland/PV74/95	H7N7	tyIE95	Low	AF028021
A/ostrich/Zimbabwe/222/96	H7N1	osZW96	Low	**AF202234**
A/ostrich/South Africa/M320/96	H7N7	osZA96	Low	**AF202253**
A/England/268/96	H7N7	En96	Low	AF028020
A/turkey/Northern Ireland/VF-1545 C5/98	H7N7	tyNI98	Low	**AF202246**
A/peregrine falcon/U.A.E./188/2384/98	H7N3	pfAE98	High	**AF202254**
A/teal/Taiwan/WB2-37-2TPFE2/98	H7N1	tlTW98	ND[b]	**AF202256**
A/chicken/Italy/1081/99	H7N1	ckIT99	Low	**AF202255**

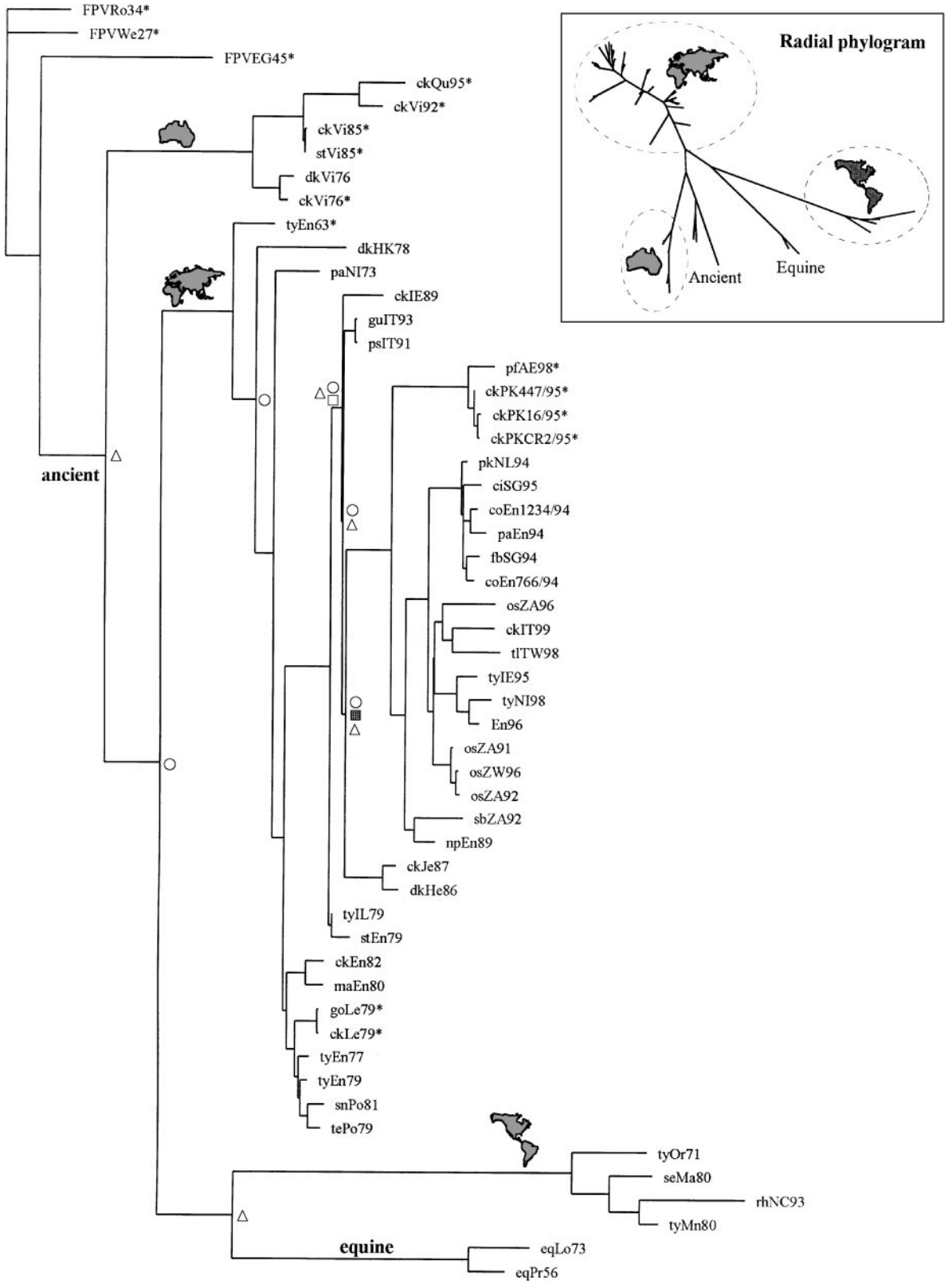
[a]Pathogenicity determined in in vivo tests
[b]Not determined
[c]Sequence determined from a cDNA clone
[d]Viruses with accession numbers in bold were sequenced in this study

and 1995. The H7N3 viruses were quite distinct from those of the same subtype combination isolated in Pakistan in 1995 (Fig. 1).

The earliest isolate in the Eurasian lineage is the HPAI virus tyEn63, followed by the non-pathogenic viruses dkHK78 and paNI73. These isolates are distinct from each other by maximum likelihood and maximum parsimony but by neighbor joining are included within the clade of viruses isolated between 1977–1982. Several isolations of H7 AI viruses were made in this period during surveillance studies in Germany [29], investigations of respiratory disease in turkeys in Israel [20], and investigations of HPAI outbreaks in the UK [4]. The percentage homology scores for this group of eight viruses (excluding tyEng63, dkHK78 and paNI73) range from 96.7 to 99.4 for amino acids and from 95.6 to 99.8 for nucleotides and the clade contains both HPAI and low pathogenicity viruses. Viruses stEn79 (from an African starling in quarantine in the UK after import from South Africa) and tyIL79 were also isolated in 1979 but are clearly distinguishable from the English and German viruses.

The next three nodes along the Eurasian branch do not have significant probabilities and this is reflected in the maximum parsimony and neighbor joining trees. In addition the distances between the nodes are extremely short and it appears most likely that the viruses on these branches have diverged from a common ancestor. Perhaps surprisingly the sources of these isolates are quite different, being obtained from a duck and chicken in Germany in 1986 and 1987

Fig. 1. Phylogenetic relationships among influenza A virus isolates of H7 subtype. The results are presented as an unrooted maximum likelihood phylogram, where the branch lengths are proportional to the nucleotide differences between the sequences. *HPAI, Major branches with low significance: Δ Neighbor joining, ○ Maximum parsimony, □ Maximum likelihood, ■ Maximum likelihood $P > 0.05$ significance


FPVRo34*
FPVWe27*
FPVEG45*
ckQu95*
ckVi92*
ckVi85*
stVi85*
dkVi76
ckVi76*
tyEn63*
dkHK78
paNI73
ckIE89
guIT93
psIT91
pfAE98*
ckPK447/95*
ckPK16/95*
ckPKCR2/95*
pkNL94
ciSG95
coEn1234/94
paEn94
fbSG94
coEn766/94
osZA96
ckIT99
tlTW98
tyIE95
tyNI98
En96
osZA91
osZW96
osZA92
sbZA92
npEn89
ckJe87
dkHe86
tyIL79
stEn79
ckEn82
maEn80
goLe79*
ckLe79*
tyEn77
tyEn79
snPo81
tePo79
tyOr71
seMa80
rhNC93
tyMn80
equine
eqLo73
eqPr56
ancient
Radial phylogram
Ancient
Equine

respectively, chickens in Ireland in 1989, a psittacine bird in quarantine in England in 1991 and a gull in Italy in 1993. It is probable that isolates stEn79 and tyIL79 are also related to this ancestor.

A cluster of H7N3 HPAI viruses is separated from the other Eurasian isolates by a relatively long branch, indicating that the viruses in this group are either diverging into a separate lineage, or have a different origin. Three of these viruses were obtained from chickens in 1995 during HPAI outbreaks in Pakistan [24] and the other was isolated in 1998 from a peregrine falcon in the UAE (pfAE98, the first avian influenza isolate from a raptor). The cleavage site amino acid motifs of the HPAI isolates from Pakistan show an unusual degree of variation; ckPK447/95 and ckPKcr2/95 were reported earlier as being different, with the motifs PETPKRKRKRGLF and PETPKRRKRGLF respectively [5] and isolate ckPk16/95 shows further variation in this motif with a novel cleavage site of PETPKRRNRGLF which has not been described previously. The cleavage site of the peregrine falcon isolate has only one silent nucleotide change compared with ckPKcr2/95. There is greater than 99.4% amino acid and 99.6% nucleic acid homology between the Pakistan isolates, and greater than 98.5% amino acid and 97.7% nucleic acid homology with pfAE98.

Next in the lineage is a discreet pair of isolates, sbZA92 and a virus from birds in quarantine in England (npEn89). The remaining 15 viruses isolated between 1991 and 1999 diverge from a common origin with clear groups being distinguishable. Of the four isolates from ostriches in southern Africa three; osZW96, osZA91 and osZA92 have high genetic relatedness. The most recent isolate from ostriches, in South Africa, osZA96, shows some divergence from these and appears closer to the 1998 wild duck isolate from Taiwan and the 1999 H7N1 isolate from poultry in Italy. Close to these viruses is a distinct group of three viruses consisting of the human eye H7N7 isolate, En96, and tyIE95, described by Banks et al [7], and tyNI98 which has closer nucleotide homology with the virus isolated from the eye of a patient virus (98.6%) than the earlier Irish turkey isolate (98.2%).

The last group, which includes pkNL94, is comprised of six H7N1 viruses from caged birds in England, The Netherlands and Singapore and isolated over a one and a half-year period during 1994–1995. These viruses have homology scores in the range of 96.7% to 98.9% for amino acids and 97.6% to 98.8% for nucleic acids.

Recently it has been suggested that additional glycosylation of the HA together with a shortened NA stalk are characteristic features of the H5 and H7 chicken viruses [22]. The authors also observed that all the avian H7 viruses with additional glycosylation sites were highly pathogenic and suggested that additional glycosylation may precede the development of pathogenicity of the influenza viruses during their evolution in chickens. Inspection of the sequences used in the present study shows that of the fifty four isolates nine have additional glycosylation sequons on the HA1 globular head (Table 2). Of these six are HPAI viruses, five isolated from chickens (FPVWe27, FPVRo34, FPVEG45, ckVi76, ckLe79) and one isolated from turkeys (tyEn63), and three are low pathogenicity

Table 2. Viruses with additional glycosylation sequons at the HA1 globular head

	Position of additional glycosylation sequons (H7 numbering)		
	123	149	188
A/FPV/Weybridge/27[a]	+		
A/FPV/Rostock/34[a]	+	+	
A/FPV/Egypt/45[a]	+		
A/equine/Prague/1/56		+	
A/turkey/England/63[a]	+		
A/chicken/Victoria/76[a]			+
A/chicken/Leipzig/79[a]	+		
A/seal/Mass/1/80	+		
A/chicken/Italy/1081/99	+		

+: sequon present
[a]HPAI viruses

viruses isolated from chickens (ckIT99), horses (eqPr56) and a seal (seMa80). Nine HPAI viruses do not possess additional glycosylation sites, six isolated from chickens (ckVi85, ckVi92, ckQu95, ckPK447/95, ckPKCR2/95, ckPK16/95) and three from other species: African starling (stVi85), peregrine falcon (pfAE98), goose (goLe79). Thus additional glycosylation at the globular head of HA1 does not correlate conclusively with high pathogenicity, or with isolation from chickens [6].

Although we have examined considerably more viruses than Rohm et al. [26], the major phylogenetic findings were essentially similar to those of the earlier study. The data obtained agree with their important observation that HPAI viruses do not constitute a separate phylogenetic lineage or lineages, but appear to arise from non-pathogenic strains. This is supported by the in vitro selection of mutants virulent for chickens from an avirulent H7 virus [16]. These findings are in keeping with the theories of the molecular basis for the mutation of avian influenza subtype H5 and H7 viruses from low to high virulence in poultry put forward by Garcia et al. [11] which represents an extremely important consideration in the control of HPAI. Rohm et al. [26] also considered that their data indicated that the equine H7 viruses emerged by divergence from the Eurasian lineage. However in the present study this does not appear to be so clear cut. Inspection of the various phylogenetic trees produced using different algorithms and either unrooted or rooted to an H10 sequence leads us to the conclusion that the equine viruses, which are all genetically quite closely related [12], are equally as likely to have shared a common ancestor with viruses of the North American lineage. The booststrapping values for the nodes where the sequences of these viruses diverge are of low predicted significance.

During the severe winter of 1979 in England and on the continent of Europe, a number of H7 subtype avian influenza viruses were isolated. In Norfolk, England

H7 influenza A viruses of high and low virulence for chickens were isolated from six turkey farms in combination with N2, N3, or N7 [4]. Contemporaneously, an H7N7 influenza A virus was isolated near Leipzig, Germany, from chickens that had developed signs characteristic of infection with HPAI [27]. Within 20 miles of this outbreak in chickens three H7N7 viruses were isolated from domestic geese [27]. In Belgium two avirulent influenza viruses of H7N7 subtype were isolated in 1980; one was from hens that were showing a drop in egg production and the other from broilers with a respiratory disorder [23]. In addition, surveillance studies on feral waterfowl in Europe around this time yielded several isolates of subtype H7 [14, 25, 28, 29]. An outbreak of respiratory disease in turkeys on a farm in Ramon, Israel was also investigated in 1979 and resulted in the isolation of an H7N2 influenza A virus [20]. An influenza virus of the same subtype was isolated from mallards found dead on a nearby reservoir [18]. These Israeli turkey and duck isolates were reported to be antigenically similar in their reactions to a panel of five monoclonal antibodies, but an H7N7 virus isolated from starlings found dead near Lake Kinneret, Israel in February 1978 was found to be antigenically quite different [17]. None of these isolates was highly pathogenic for chickens [19]. H7 influenza A virus isolations at this time were not restricted to the European continent and Israel and examples of the many surveys and viruses isolated around this time have been reviewed [1, 21].

The isolation of a relatively large number of H7 subtype avian influenza viruses of both high and low virulence in Europe during a very short period at the end of the 1970s and beginning of the 1980s is unusual. Alexander and Spackman [4] suggested that the isolations in England in 1979, which were exclusively in Norfolk, an area on the migratory route of numerous species of waterfowl, may have occurred as a direct result of unusual bird movements following a harsh winter and unusual weather patterns in the spring. In addition to disrupted waterfowl migration throughout Europe there were reports of huge flocks of starlings and other birds that may have invaded poultry houses and spread influenza viruses directly. Phylogenetic analysis of the representative viruses used in the present study indicates that English isolates tyEn77 and tyEn79 were closely related to the German wild bird isolates tePo79 and snPo81 and that the HPAI German isolates goLe79 and ckLe79 appear to have emerged from the same close common ancestor as these viruses of low virulence. The 1980 isolate from a macaw, maEn80, and the 1982 chicken isolate, ckEn82, both from England also showed close relationships to this group. In contrast the two 1979 isolates tyIL79 and stEn79, although close to each other were distinct from the England/German 1979/80 isolates. These findings suggest that more than one lineage of H7 subtype was circulating in Europe and the middle east during this period.

The extremely close relationship of the 3 HPAI Pakistan H7N3 isolates made in 1995 (ckPK447/95, ckPK16/95 and ckPKCR2/95) and the HPAI isolate of the same subtype from a peregrine falcon in UAE in 1998 (pfAE98) poses an epidemiological puzzle. There have been no reports of HPAI H7N3 viruses since the outbreaks in Pakistan in 1994/95 where the last outbreak was recorded in August 1995 [24]. It is common for hobby birds from the UAE to be flown in

Pakistan, but it was reported that the affected bird had not. However, it had flown in Syria six weeks before clinical signs were seen. Possibly, the bird had been in contact with birds from Pakistan, but this would presuppose the virus had remained present in birds in the area between 1995 and 1998.

The human eye isolate, En96, obtained from a woman who kept ducks that had close contact with wild waterfowl [7], has closer genetic relatedness to the more recently isolated tyNI98, a representative from the viruses infecting poultry on the island of Ireland in 1998, than the 1995 isolate tyEI95. This may indicate that viruses of the H7N7 subtype are currently circulating in the wild bird population of the British Isles and that they are responsible for periodic introductions to domestic poultry. Although these are not HPAI viruses they have nevertheless been associated with increased mortality and morbidity in the affected poultry flocks, and there is always the possibility that a highly pathogenic form might emerge.

It is unclear what the role of commercially reared ostriches may be in the dissemination of AI viruses and it is not possible from the results of this study to determine whether the ostriches are a reservoir for these viruses or if they have become infected through contact with wild waterfowl. The latter is more likely, due to the practice of raising ostriches on open ranges.

The international captive bird trade, especially when involving air transportation, represents a potentially important method by which influenza viruses may be disseminated rapidly around the world. Since the mid 1970s birds dying in quarantine in Great Britain or captive birds dying in transit at London (Heathrow) Airport, have been examined for influenza viruses. Although numerous avian influenza viruses have been isolated from this source [2, 3], to date only two have been of the H7 subtype, stEn79 (H7N7) and npEn89 (H7N7). In total the International Reference Laboratory for avian influenza at Weybridge, UK has received eleven H7 subtype isolates, all of low virulence for chickens, from cage birds since 1973. Six of these were submitted between 1994 and 1995 from England, The Netherlands and Singapore, all six have N1 neuraminidase. Four isolates were from privately owned aviaries (not quarantine) which had suffered mortalities in conures, parrots and parakeets [8]. The other two viruses were isolated from a fairy bluebird destined for export from Singapore, and a common iora confiscated for being imported illegally from Indonesia to Singapore. The phylogenetic results demonstrate that these six isolates have a common origin even though they were isolated from distant geographical regions and, in particular, highlights the risk that the illegal cage bird trade poses for the introduction and dissemination of potentially pathogenic avian influenza viruses throughout the world. However, the wide distribution of the isolates from caged birds on different branches of the Eurasian lineage indicates that influenza viruses are probably not perpetuated in this type of bird for long periods.

Phylogenetic analysis of avian influenza viruses represents an important tool in the understanding of the origins of specific isolates and the epidemiology and spread of disease. The present study highlights the several lineages and sublineages of H7 influenza viruses and the apparent geographical and temporal

influences, which appear to be more important than those of the host species infected. In addition, the consistency of the haemagglutinin cleavage site motif in viruses of low virulence for poultry and the shared phylogenetic branches by viruses of both high and low virulence support the theory that HPAI viruses arise by mutation once they have spread to poultry and are not maintained as a separate entity in waterfowl or other birds.

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