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Characterisation of an avian influenza A virus isolated from a human – is an intermediate host necessary for the emergence of pandemic influenza viruses?

Brief Report

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Summary. The partial sequencing of the internal and the neuraminidase genes of isolate 268/96 obtained from a woman with conjunctivitis showed all seven to have closest homology with avian influenza viruses. The entire nucleotide sequence of the haemagglutinin gene of 268/96 had close, 98.2%, homology with an H7N7 virus isolated from turkeys in Ireland in 1995. This appears to be the first reported case of isolation of an influenza A virus from a human being infected as a result of direct natural transmission of an avian influenza virus from birds.

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Pandemics of influenza A infections arise every few decades and usually have a devastating effect on the human population. They occur as a result of "antigenic shift" i.e. when an influenza A virus of different antigenic subtype to those present in the population suddenly emerges. In the 20th Century there have been four identified pandemics of influenza, beginning in 1918 caused by a virus of H1N1 subtype, 1957 due to H2N2 subtype virus, in 1968 due to H3N2 subtype virus and 1977 due to the re-emergence of H1N1 virus.

Demonstration that the 1968 H3N2 pandemic virus differed from the H2N2 virus, present from 1957 to 1968, in the substitution of two genes, PB1 and the important surface glycoprotein haemagglutinin (H) gene, with genes almost certainly from an influenza virus of avian origin, led to the suggestion that antigenic shift occurred as a result of reassortment of genes in dual infections with viruses of human and avian origin [6, 9] resulting in the emergence of viruses with sufficient genes from the virus of human origin to allow replication and spread in the population, but with a different haemagglutinin surface glycoprotein so that the human population could be regarded as immunologically naive. Systematic surveillance

studies into the presence of influenza viruses in avian species, undertaken in response to this finding, revealed enormous pools of influenza A viruses in wild birds, especially migratory waterfowl [17]. In addition, unlike mammals where the number of subtypes present appears to be limited, all 15 H and 9 N subtypes recognised currently have been recorded in birds in most possible combinations.

Pigs have been suggested as "mixing vessels" in which reassortment between human and avian influenza viruses could take place with the emergence of viruses with sufficient genes from the virus of human origin to allow replication and spread in the human population, but with a different haemagglutinin surface glycoprotein [15]. The concept of an intermediary host was necessary because, although in volunteer experiments transitory infections of humans with avian influenza viruses had been established, no report of natural infection of humans had been substantiated [2]. This theory has some attractions as pandemic influenza has usually appeared to spread from an epicentre in areas of Asia where high concentrations of humans, pigs and waterfowl live in close proximity [16]. However, pigs have been shown to be susceptible to infection with viruses representative of each of the H subtypes of influenza A viruses [10] and it is therefore surprising that natural infections of pigs have been restricted to viruses of H1 and H3 subtypes, although isolates of these subtypes have been typical of human, avian or pig viruses.

Kurtz et al. [11] considered the source of the virus, A/England/268/96 (H7N7), isolated from the eye of a 43-year-old woman with conjunctivitis, to be waterfowl as she tended a collection of 26 ducks of various breeds which mixed freely with feral waterfowl on a small lake. However, H7N7 viruses have been isolated from mammals, being responsible for equine-1 influenza, although no isolate of H7N7 virus has been made from horses for 15 years [18], and an outbreak of disease in harbour seals in 1979–1980 [21]. The latter virus was transmitted to one of the seal handlers causing conjunctivitis [20]. We report that partial nucleotide sequencing of all eight genes of 268/96 virus indicates that each is closest to genes of influenza viruses of avian origin and that the nucleotide sequence of the entire H gene shows close homology with an H7N7 isolate from turkeys in Ireland in 1995.

The origins of the genes of virus 268/96 were investigated by reverse transcription of viral RNA, amplification by PCR and determination of the nucleotide sequences by the dideoxynucleotide chain termination method [14]. The entire sequence of the H gene of 268/96 was obtained from both the original isolate grown in primary rhesus monkey kidney cells and after one passage in embryonated fowls' eggs (Genbank accession number AF028020) and partial nucleotide sequences of the other genes from egg passage 1 virus. The entire sequence of the H gene of the closest avian H7N7 isolate available, both temporally and geographically, A/turkey/Ireland/PV74/95, was also obtained from virus passaged once in eggs (Genbank accession number AF028021).

The methods for RT/PCR of the H and N [23], or internal genes [1] were as described. DNA sequencing was done using the Thermo Sequenase radiolabelled terminator cycle sequencing kit (Amersham).

Primers for RT/PCR and sequencing the H were: mRNA sense – Vgenex [22] 5′-AGCAAAAGCAGG-3′, H7.5 5′- AGCAAAAGCAGGGGATACAAAATG-3′, H7.6 5′,-ACATAYAGYGGRATAAGRACYAATGG-3′, H7.8 5′-GARCAGAC-CAAAYTATATGG-3′, J5 5′-GCAGGGCAGTGGGCAAATGTCC-3′, H7.3 5′-GCACYCARTCRGCAATTGATCA-3′, H7.10 5′-GARGARGATGGYACTGG-TTG-3′, H7.11 5′-TGGTTTAGCTTSGGGGCRTCATG-3′. Complimentary to mRNA sense-H7.15′-AATTGGTCACATTGRGGTGG-3′, H7.2 5′-GCATAGA-ATGAAGAHCCTGATCT-3′, H7.9 5′-TVAAAGTVACTGTGTCATTGG-3′, H7.7 5′-CYTCYCCYTGTGCATTTTGATG-3′, H7.4 5′-GCCATACADTRCTC-ATCACA-3′, Cgenex [22] 5′-ACGCGTGATCAGTAGAAACAAGG-3′. Primers for RT/PCR and partial sequencing of the N gene were: N7 5′-TGAATCCTAAT-CAAAAACT-3′ and N20c 5′-GGATCGCATGACACATAAGG-3′. Primers for RT/PCR and sequencing the internal protein genes were as described [1]. Phylogenetic analysis was done using the PHYLIP phylogenetic inference package (version 3.5c) [7] and results presented as an unrooted maximum likelihood tree of the entire nucleotide sequences of the H gene coding region, excluding the cleavage site sequences, in which the branch lengths are proportional to the nucleotide differences between the sequences (Fig. 1).

The partial sequences of the seven genes coding for the internal proteins and the neuraminidase of 268/96 were analysed by a ‘best-local-homology’ rapid search procedure of a gene database. The two closest homology viruses for each gene (Table 1) were isolates from avian species with the exception of the closest homology for the PA gene with A/swine/Hong Kong/126/82, the second closest homology with the NP gene with A/mink/Sweden/84 and the second closest homology with the PB1 gene with A/Nt/60/68. However, the swine and mink isolates are thought to have been the results of direct spread from birds [3, 13] and the PB1 gene of the 1968 pandemic virus has been shown to have been derived from avian influenza virus [9].

Comparison of the nucleotide sequences of the entire H genes of 268/96 and ty/PV74/95 gave an homology score of 98.2%. In the sequence of 1732 nucleotides there were 32 differences which coded for 11 amino acid differences. No nucleotide differences were seen between tissue culture grown and egg passage 1 268/96 virus.

The entire H gene sequences of 14 other H7 viruses were available from databank sources. Phylogenetic analysis of these and the two sequences obtained in the present study confirmed the extremely close relationship of 268/96 and ty/PV74/95 compared to the other viruses (Fig. 1). The sequence for 268/96 was quite distinct from those of the equine-1 or seal/Massachusetts/80 viruses.

The deduced amino acid sequences around the receptor binding site of the haemagglutinin [12] were identical for 268/96 and ty/PV74/95 and are typical of those seen in H7 avian influenza viruses. This would indicate that, like most avian influenza viruses, they bind preferentially to the N-acetylneuraminic acid-α2, 3,-galactose linkage, whilst human influenza viruses bind the NeuAc-α2, 6Gal linkage on sialyloligosaccharides [8]. This did not appear to prevent replication

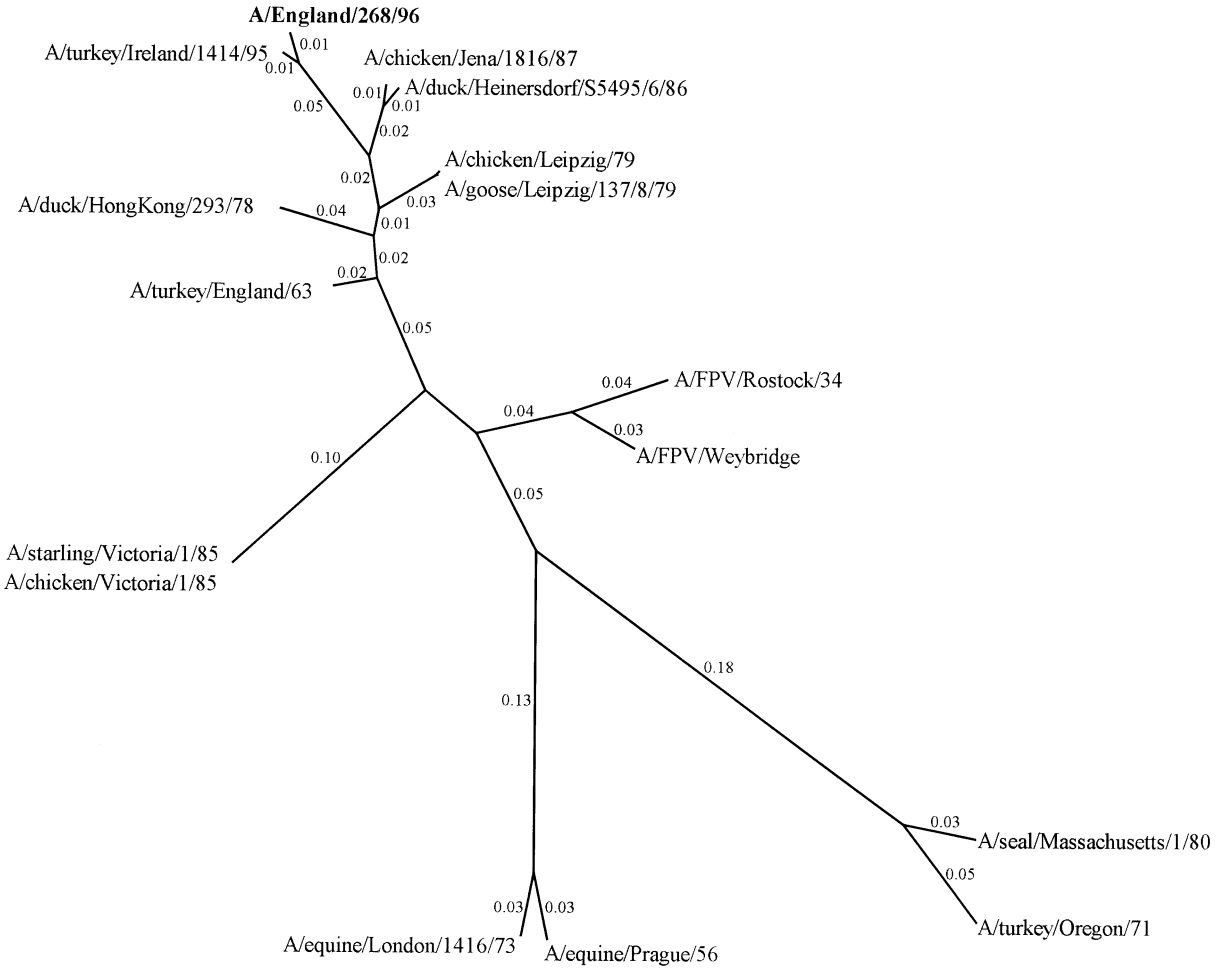

Fig. 1. Phylogenetic relationships among influenza A virus isolates of H7 subtype

of 268/96 in the conjunctiva, but would have probably contributed to the limited invasiveness of the infection in the patient.

The close phylogenetic relationship between 268/96 and ty/PV74/95 is in keeping with the assumed introduction of the H7N7 viruses to the turkey flock in Ireland and the duck flock tended by the patient from feral birds. There is no evidence to suggest, nor any reason to assume, that the 268/96 virus passed through an intermediate host. This therefore appears to be the first reported case of isolation of an influenza A virus from a human being infected as a result of direct natural transmission of an avian influenza virus from birds. The infection in this case appears to have been restricted to replication in the conjunctiva but there is no reason to suppose that, under the right conditions, the virus could not have been passed on to other humans. Certain pig influenza isolates have been shown to replicate only in the upper respiratory tract of infected pigs inducing no humoral immune response, but in experiments are transmissible to susceptible

Table 1. Homology of A/England/268/96 (H7N7) with other influenza viruses

Gene	Match rank	Probe sequence	Virus matched	Nucleotides matched	Nucleotides mismatched	Homology %
PB2	1	14–179	A/gull/Maryland/704/77	162	4	97.5
	2		A/gull/Astrakhan/227/84	161	5	97.0
PB1	1	40–287	A/turkey/Minnesota/833/80	232	16	93.5
	2		A/Nt/60/68	230	18	92.2
PA	1	43–302	A/swine/HK/126/82	243	17	93.0
	2		A/ruddy turnstone/NJ/47/85	242	18	92.6
NP	1	43–252	A/mallard/Astrakhan/244/82	207	3	98.6
	2		A/mink/Sweden/84	202	8	96.0
N	1	78–310	A/chicken/Germany/49	203	30	87.1
	2		A/FPV/Weybridge	190	43	81.5
M	1	40–285	A/shearwater/Australia/172	243	3	98.8
	2		A/duck/Czech/56	242	4	98.3
NS	1	42–255	A/mallard/New York/675/78	208	6	97.2
	2		A/mynah/Haneda-Thailand/76	208	6	97.2

pigs placed in contact [4]. The main danger to the human population of the direct spread of influenza viruses from avian hosts would not appear to be from the wholly avian virus, but if the initial human host or those infected subsequently are infected concurrently with human influenza virus reassortment could lead to antigenic shift and the emergence of pandemic virus.

The most popular of the current theories on the origins of pandemic influenza revolves around the pig as a mixing vessel for avian and human influenza viruses [19]. However, there are some anomalies associated with this theory, such as why so few influenza subtypes have been recorded in natural infections of pigs and why, since human and avian viruses infect pigs so readily, pandemics occur so infrequently? Infection of humans directly with influenza viruses from avian sources must occur only rarely and the chance of dual infection and reassortment must be substantially rarer. Nevertheless, the extremely low probability of pandemic influenza viruses arising from direct infection of humans with virus from an avian species could be regarded as consistent with the number of years between each emergence of pandemic influenza virus. During the preparation of this paper a preliminary report on the isolation of an H5N1 virus from a three-year-old child in Hong Kong who died was published [5].

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