Molecular epidemiology of influenza viruses: Memorandum from a WHO Meeting*

This Memorandum summarizes recent information on influenza viruses of non-human hosts, discussed at the WHO Consultation on Molecular Epidemiology of Influenza Viruses, Athens, Georgia, USA, in September 1986. It was noted that a wide variety of antigenic variants of influenza viruses have been isolated from non-human hosts, especially birds. Of particular epidemiological interest in recent years has been the isolation of H7N7 influenza viruses from epidemics of disease in seals, the isolation of an H10N7 virus from outbreaks of disease among domestic mink, and the occurrence of pathogenic avian influenza viruses in Australia, Ireland and the USA.

Of particular epidemiological interest in recent years has been the isolation of H7N7 influenza viruses from seals, associated with high mortality, the isolation of an H10N7 virus from mink that also caused high mortality, and the isolation of highly pathogenic avian influenza viruses in Australia, Ireland and the USA. The present report reviews the current status of knowledge of influenza in animals. Because of several incidents in recent years of transmission of avian influenza viruses to mammalian species, it was important to review information available on the virological and molecular aspects of these events.

HOST RANGE OF INFLUENZA VIRUSES

Influenza in swine

The H1N1 and H3N2 influenza viruses, closely similar to those currently circulating in humans, have been isolated from pigs. Classical swine H1N1 influenza virus, clearly distinguishable from human viruses, continues to circulate in pigs in North America whereas it has been proposed that the causative viruses of swine influenza may have originated in birds. The reappearance of swine influenza in the United Kingdom is noteworthy. There is continuing speculation that the pig is an important intermediate host in host range variation, especially between humans and avians.

Serological studies using both haemagglutination inhibition (HI) and single radial haemolysis (SRH) on pig sera from China frequently show antibody to influenza A (H3N2) and (H1N1) viruses and less frequently antibody to influenza B and C viruses. The unexpected detection of antibodies to H2N2 viruses, which had not been found in pig sera before 1985, was demonstrated by HI and SRH and were confirmed by neuraminidase inhibition (NI) assays. Influenza viruses of the H3N2, H1N1 subtypes of influenza A and influenza C viruses have been isolated from pigs in China, mainly in the winter and spring. The H3N2 strains from pigs have the ts” phenotype, while the H1N1 strains and influenza C viruses are ts”.

Influenza viruses of the H3N2 subtype, antigenically similar to A/Port Chalmers/1/73, have been isolated in the last three years from swine in Belgium
and France and more recently in Ireland. Sequence analyses of the haemagglutinins (HA) of representative viruses indicate that, although antigenically similar, the haemagglutinins were quite distinct. In the HA glycopolypeptide, 15 amino acid substitutions compared with the sequence of A/Victoria/3/75 haemagglutinin were detected, 13 of which have not been seen to change in the haemagglutinins of viruses isolated from humans. Overall, nucleotide substitutions of 6.7% compared with A/Victoria/3/75 including about 2.5% silent mutations indicate similar degrees of change to those observed in viruses from humans over the last 10 years.

**Influenza in horses**

Two subtypes of influenza, equine 1 (H7N7) and equine 2 (H3N8), continue to circulate in horses in the world. In recent years, equine 1 (H7N7) influenza viruses have not been received at the WHO collaborating laboratories. The possibility was that this virus may have disappeared from horses. An international questionnaire established that the last equine H7N7 virus isolate was in Mongolia in 1980 and that antibodies were detectable in young non-vaccinated horses in Asia and central Europe up to the present time. In Czechoslovakia, in 1985, antibody to the equine 1 (H7N7) virus has been demonstrated in 25 out of 42 horse sera (unvaccinated horses, mostly foals, HI titre 1:128–1024). These studies indicate that equine 1 (H7N7) is still circulating in the world in a very benign form.

Partial sequencing of the HA gene of 11 equine 1 influenza viruses isolated between 1956 and 1977 showed evidence of heterogeneity. From the nucleotide sequence, it was calculated that the mutation rate in HA1 of equine H7N7 for all years except 1963–64 was of the order of 0.17%, whereas in 1963–64 it was 3.6% per year. This indicated the possibility of the introduction of a different H7 gene in 1963, possibly from an avian source.

The H3N8 influenza viruses isolated from horses in 1985 in Europe and the Americas were antigenically very similar to A/Fontainebleau/79. By sequence analyses, up to 7 amino acid substitutions in HA1 compared with A/Fontainebleau/79 were detected, supporting the antigenicity results and suggesting a continuation of the A/Fontainebleau/79 to A/Kentucky/81 drift.

**Influenza in birds**

Influenza A viruses of all known subtypes can be isolated in aquatic birds of the world, particularly ducks. In recent years, three highly virulent influenza viruses appeared in domestic poultry in different parts of the world. In Pennsylvania (USA) an avirulent H5N2 influenza virus appeared in chickens in April 1983 and subsequently became highly virulent in November 1983. Acquisition of virulence was associated with a single point mutation in the HA gene that abolished a carbohydrate side-chain in the vicinity of the connecting peptide.

In Ireland a highly virulent H5N8 virus appeared in turkeys and an antigenically identical virus was isolated from a nearby duck farm where it caused no deaths. This virus acquired high virulence simply by transfer to the domestic turkey. In 1985 in Australia, a highly virulent H7N7 virus appeared in chickens; the virus was also isolated from a starling. Each of these epidemics probably originated from wild birds and shows the potential of the influenza gene pool in nature.

Studies in wild ducks in different countries (China and USA) continued to demonstrate an extensive gene pool of different influenza viruses. Of particular interest were studies showing that influenza viruses of multiple subtypes can be isolated from mallard ducks on a city pond in Madison (WI, USA) every month of the year. Previous studies left some doubt as to how these viruses were maintained during the winter and spring months of the year.

A systematic study of influenza viruses in sea and shore birds in the USA is in progress; initial results indicate a high incidence of different subtypes of influenza viruses in a range of sea birds in May of each year.

**Influenza in other species**

Seals. There have been two disease outbreaks in harbour seals (Phoca vitulina) involving influenza viruses. The first occurred in 1979–80 and approximately 20% of the harbour seal population of the northeast coast of the USA died of severe respiratory infection with consolidation of the lungs, typical of primary viral pneumonia. Influenza virus of the H7N7 subtype was recovered from the lungs and brains of dead seals. This is antigenically related to the fowl plague virus, A/FPV/Dutch/27 (H7N7), a virulent influenza virus of chickens not previously found in mammals. The virus preferentially replicated in mammals and caused conjunctivitis in humans.

A second influenza virus was isolated from harbour seals in 1982. Influenza A viruses of the H4N5 subtype (which had previously been detected only in birds) were recovered from harbour seals dying of viral pneumonia on the New England coast from June 1982 till August 1983. RNA–RNA hybridization studies indicated that each of these viruses originated from birds and provide evidence for avian to mammalian transfer of influenza viruses.
Whales. Influenza A viruses of the H1N3N2 and H1N3N9 subtypes were isolated from the lung and hilar node of a pilot whale (Globicephala melas) in 1984. Serological, molecular, and biological analyses indicate that the whale isolates are closely related to the H13 influenza viruses from gulls. Recent studies have detected a third influenza virus in the lungs of the same animal antigenically. Preliminary studies suggest that the haemagglutinin may constitute a new subtype (H7N5).

Mink. Influenza viruses of the H10N7 subtype have been isolated from mink. This virus caused high mortality in this species and had previously only been isolated from avian species. Molecular characterization of this virus is in progress.

MOLECULAR BASIS OF THE HOST RANGE OF INFLUENZA VIRUS

Viral genes involved in the host range

The viral "gene" involved in host range variation in influenza virus has not been established; recent evidence suggests the involvement of RNA segment 5 nucleoprotein (NP gene) in species specificity of H3N2 strains. Temperature-sensitive (ts) mutants of A/FPV/Rostock/34 (H7N1) with a defect in the NP gene cannot be rescued by double infection with human H3N2 viruses, while all avian H3N2 strains are able to rescue these mutants. Pig isolates of H3N2 influenza virus from Hong Kong can be divided into two groups in this respect. Viruses of one group contain an "avian-like" NP, while the other swine isolates contain "human-like" NP. From these and other data it is suggested that human H3N2 viruses cannot be transmitted directly to birds without prior adaptation to or reassortment in pigs.

Influence of host cells on virus selection

The nucleoproteins (NPs) of influenza A viruses are phosphoproteins. The phosphopeptide fingerprints of the NPs are specific for each virus strain when grown in the same cells, and they also depend on the host cell. When the NP gene of fowl plague virus is replaced by that of the Hong Kong strain, the host range is changed.

In addition to antigenic drift and shift, there is evidence for another mechanism of variation for influenza viruses. This mechanism, which is independent of the immunological pressures that provoke antigenic drift, is a result of the selection of virus subpopulations by the host cell in which virus is cultivated. Adaptation of influenza viruses to growth in embryonated eggs resulted in selection of variants which were antigenically and biologically distinguish-
the A/Ann Arbor/6/60 cold-adapted virus. Candidate vaccine strains should be selected on the basis of having six internal genes from the mutant donor parent. This offers two theoretical advantages: (1) live candidate vaccine strains that have a constant gene constellation (except for their HA and NA) might be expected to have uniform levels of attenuation, and (2) there would be a lower rate of reversion to virulence for the ca mutant genes since there are multiple mutations throughout the genome. However, the finding of only a limited number of coding changes throughout the genome was unexpected, with a potential for revertants to occur; it is therefore important to continue to monitor the genetic stability of ca vaccines carefully.

Avian–human reassortant viruses have a larger number of coding changes in the internal genes donated by the avian parent and provide the possibility of greater genetic stability. Reassortant vaccine viruses have been produced by mating an avian influenza A virus with a human influenza A virus. The avian–human reassortant influenza A viruses that contain the human HA and NA genes and each of the other genes from the avian parent have been shown to reproducibly confer the attenuation phenotype on human H3N2 and H1N1 viruses. These reassortants (1) are infectious and immunogenic; (2) induce protective immunity to experimental challenge; (3) are non transmissible; and (4) are stable genetically. Genetic studies indicate that two or more genes contribute to their attenuation for primates.

STRUCTURAL AND FUNCTIONAL DOMAINS ON VIRAL PROTEINS

The neuraminidase

The three-dimensional structure of N9 neuraminidase heads has been established at 3Å resolution. When the amino acid sequences of the N9 and N2 neuraminidase heads are aligned to maximize homology, there are 204 differences out of a total sequence of 390 residues. Nevertheless, the three-dimensional structure of N9 neuraminidase from an avian influenza virus, determined by X-ray crystallography at 3Å, shows the polypeptide to be folded in a similar way to that of neuraminidase of subtype N2 isolated from human influenza viruses. Small differences in the way in which the subunits are organized around the molecular fourfold axis have been observed. Crystals of antibodies complexed with N9 neuraminidase have recently been reported. Crystals have been grown of Fab fragments of monoclonal antibody NC41, complexed with influenza virus neuraminidase of the N9 subtype. Determination of the three-dimensional structure of the complexes and of the neuraminidase variants will reveal precisely how antibodies recognize the influenza virus and how the virus changes to escape this recognition.

Variability of human and animal influenza viruses

The haemagglutinin of H3 influenza viruses that had been isolated from migratory ducks in one geographic area on the Pacific flyway in Japan from 1977 to 1985 were analysed antigenically and genetically. Antigenic analysis using monoclonal antibodies to the haemagglutinins of A/Aichi/2/68 (H3N2) and A/Duck/Hokkaido/8/80 (H3N8) viruses showed that in the haemagglutinin of human strains antigenic drift occurred extensively, whereas in that of duck viruses, antigenicity was highly conserved. Nucleotide sequence analysis of the gene coding for the haemagglutinin of duck isolates showed that in ducks the genes are maintained with very small mutation rates and that viruses of different families are independently cocirculating.

Antigenic heterogeneity

Antigenic and genomic heterogeneity has been reported for influenza A in studies employing RNA:RNA hybridization techniques in the USSR and German Democratic Republic.

Amantidine resistance

Amantidine-resistant variants of the highly virulent A/chicken/Pennsylvania/83 (H5N2) virus occur frequently in chickens. Amantidine-resistant strains appeared by the third day after treatment with 0.01% drug in their drinking water. The majority of these viruses are less virulent than the parent and in all studies the amantidine-resistant viruses failed to compete with the parental virus in transmission studies. The amantidine-resistant viruses appear to be biological cripples and may explain why amantidine-resistant human strains are infrequently detected.

THE MOLECULAR BASIS OF VIRULENCE

The molecular changes in the A/chicken/Pennsylvania/83 (H5N2) virus associated with acquisition of virulence were confined to the HA molecule. An amino acid change at residue 13 of HA1 was associated with cleavability of the HA molecule and virulence. Direct amino acid sequencing of tryptic peptides treated with glycosidase H showed that the virulent viruses lost a carbohydrate side-chain located in the vicinity of the cleavage site of the HA molecule between HA1 and HA2 in the three-dimensional structure by a mutation at residue 13. These studies
establish that a single point mutation in the haemagglutinin gene of the avirulent A/chicken/Pennsylvania/1/83 (H5N2) was all that was required to produce the highly virulent chicken/Pennsylvania virus.

A comparison of the amino acid sequence of the connecting peptides of avirulent and virulent H5 influenza viruses provided evidence for the role of the connecting peptide in determining virulence. Although the number of amino acids in the connecting peptide varies, all of the virulent viruses sequenced contained a series of basic amino acids at the cleavage site between HA1 and HA2. In contrast, all of the avirulent viruses examined contained a single Arg at the connecting peptide as do all the other avirulent viruses of different HA subtypes. The results suggest that the number of amino acid residues within the connecting peptides is not important but that a pair of basic amino acids at the cleavage site is required for cleavage activation of the HA molecule.

RECOMMENDATIONS

1) The World Health Organization should continue to monitor the status of influenza in lower animals and birds and to periodically update the information on the gene pool and its potential for initiating infections in humans.

2) There is only a limited amount of gene sequence information available on the influenza viruses from animals. Investigators are encouraged to sequence the genes of a number of influenza viruses with a view to providing a sufficiently large database to obtain information on the molecular mechanisms of host range, tissue tropism and virulence.

3) The molecular basis of pathogenicity of influenza viruses is still unresolved. Additional studies are needed, including the possible role of bacterial infection in exacerbation of disease.

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