Szent István University
Postgraduate School of Veterinary Science

Phylogenetic analysis of old and recent avian influenza viruses for epidemiological purpose

Thesis of PhD dissertation

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Introduction

Based on our previous 20th century epidemiologic experience it might have seemed to be a reasonable assumption that evolution of a worldwide epidemic could only be human-related, since the virus needs infected carriers, even in their latent phase, who are able to reach any part of the globe (e.g. through aviation) while carrying diseases. Whilst, we supposed this condition is not given in the case of animal epidemic. This theory was confirmed by four consecutive flu epidemics in the 20th century and the lack of their animal-related version. This highlights the significance of the avian influenza (AI) epidemic in 2003, which can be regarded as the first influenza panzootic with regard to its expansion. The epidemic was caused by a highly pathogenic (HP) H5N1 AI virus triggering severe lost among chickens, waterfowls and wildbirds. As known, the epidemic at first affected only the far eastern countries, an then subsequently spread further from China to Russia, Europe and some African countries. Since then the new virus variants has appeared in form of endemic in both the far east and geographically distant areas (e.g. Egypt, India). HP H5N1 epidemic still showed further new particular signs, e.g. earlier it never occurred that a HPAI strain could have covered such a huge and coherent area. In addition, HPAI virus was first discovered to infect the population of wild waterbirds (as natural reservoir) as well, and furthermore they became part of panzootic. The epidemic reached Hungary in February 2006 affected wild waterfowl (primarily swans). In the second and third wave it was reappeared in domesticated waterfowl farms in June 2006 and January 2007.

In this study, I have conducted genetic identification of certain representatives of the HPAI strains, and two low pathogenic (LP) viruses isolated during the H5N1 epidemic.

Due to the massive public attention triggered by the avian flu we decided to examine the archive AI viruses preserved in Animal Health Institute in Debrecen by applying the method of genetical identification. These viruses were isolated by János Tanyi in the 70's and 80's mainly from diseases affected large duck farms. Our knowledge concerning the epidemiological aspects of these cases is still quite limited. As a consequence of this fact, our aim was to find their proper place in the contemporary virus movements and to specify our knowledge in term of their epidemiological and ecological role.

Having been identified different virus subtypes in the 60's among ducks and turkeys hold in huge farms worldwide, the scientific attention turned to the importance of AIV poultry infection. However, the epidemiology of these infections was not obvious for several reasons. One of them was that several subtypes appeared in the same area or at the same time, which was inconsistent with the typical way of infection spreading caused by disease originated from one certain area. However, in the 70's the successful identification of huge variety and geographical distribution of AIV subtypes in the wild waterfowl (especially in

ducks) allowed scientists to assume that the source of poultry virus had been identified. This assumption was enhanced by the fact that the subtypes found in wild birds and in poultry often corresponded with each other in serological tests. Nevertheless, some decades later with the method of the genetic identification it turned out that subtype correspondence itself could not prove the direct epidemiologic relationship. The numerous subtypes of AI viruses identified in the population of wild waterfowl allowed to assume that this otherwise harmless virus holder has been existing for a long period, and as a result these bird species could be the natural reservoir of AIV. Currently, the poultry viruses are thought to have been originated from much earlier wild birds viruses, which refers to evolutionary relationship. According to this presumption some subtype combinations identified in poultry population is supposed to have initially arrived from a natural reservoir, but later the infection chain sustained also in poultry due to the farming condition (e.g. congestion, its special reproduction systems etc) and adaptation, which led to the development of an artificial reservoir. This also means that the maintenance of the infection chain doesn't need the transmission of new viruses from the primer reservoir. However, making such distinction was not possible that time because of lacking virus identification methods.

Objectives

In order to augment our epidemiological and evolutionary knowledge we have examined both the archive and recent AIVs to different extent. The areas taken into examination were the following:

1. To define the hemagglutinin (HA) and neuraminidase (NA) subtypes of the archive and recent viruses.
2. To determine the pathogenicity of H5 and H7 viruses based on the genetic analysis of the HA cleavage site.
3. The geographical classification of the archive viruses using phylogenetic analysis.
4. To define epidemiological connections or properties (endemic or new infections) even in the case of LP viruses.
5. To confirm or exclude the epidemic connection between the three waves of HP H5N1 epidemic.
6. To study the known markers of the genome using whole genome analysis of the H5N1, H3N8 and H7N7 strains to draw a conclusion about the epidemiology of viruses (e.g. reassortation).

Materials and methods

Origin and isolation of the viruses

The archive viruses isolated from 1969 to 1987 by Dr. János Tanyi were derived from the eastern part of Hungary from turkeys, ducks and guinea fowls. The HPAI H5N1 viruses from 13 wild bird and three domestic poultry cases in 2006/07 and two LP viruses were isolated in the Central Agricultural Office Veterinary Diagnostic Directorate in Budapest. The viruses were inoculated into the allantoic cavity of 9-11-day-old specific pathogen-free embryonated chicken eggs.

RNA extraction

Viral RNA was extracted from infective allantoic fluid using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) (archive viruses) or by High Pure Viral RNA Extraction Kit (Roche Applied Science, Mannheim, Germany) (recent viruses) according to the manufacturer's recommendations.

RNA transcription and PCR reaction

The RNA transcription and the PCR were performed in one step format using OneStep RT-PCR Kit (Qiagen) according to the manufacturer's instructions.

The HA genes of the archive strains were amplified in a nested PCR assay where the first sets of primers (published in the literature) extended the whole HA gene and the second ones a shorter target within the first product. The second sets of primers were designed with the CLC Gene Workbench 2.2.3. (CLC bio A/S, Arthus, Denmark) program. The NA and the NS genes of the old viruses and the whole genome of the new strains were amplified by published primer pairs.

DNA purification

The DNA products were gel-purified with Gel Out Kit (A&A Biotechnology, Gdynia, Poland) (archive viruses) or QIAquick Gel Extraction Kit (Qiagen) (recent viruses) according to the manufacturer's recommendations.

Sequencing

The amplicons of the archive viruses were sequenced with the same primers that were used for the RT-PCR reaction by the Agricultural Biotechnology Center (Gödöllő). The amplicons of the new viruses were sequenced with ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) also with the same primers that were used for amplification.

Genetic and phylogenetic analysis

Sequences were assembled and edited using the BioEdit 7.0.7 and the DNASTAR 7.1 (Lasergene, WI, USA) software package. Distance based neighbour-joining (implemented with the Kimura-2 parameters model) and character based maximum parsimony phylogenetic trees were generated using the MEGA 4.1 software. Potential N-linked glycosylation sites were predicted by the NetNGlyc 1.0 server.

Results

Result with the analysis of the archive strains

- I have identified and confirmed the HA and NA subtypes as well as the NS groups of the 15 archive AI isolates. While the main subtypes of ducks living in Hong Kong and the wild birds of Canada were H4, H3, H6 and H10 in Hungary H4, H5 and H10 subtypes were common at the same time.

Isolate	**Subtype (Tanyi)**	**Subtype (new result)**	**NS gene groups**
A/duck/Hung/3/70	H4	H4Nx	B
A/duck/Hung/Debr/265/70	H4	H4Nx	A
A/duck/Hung/1/235/70	H6N2	H6N2	A
A/guinea fowl/Hung/1/72	H4	H4N6	A
A/guinea fowl/Hung/2/75	H7	H7N1	A
A/duck/Hung/3/75	H4	H4N8	A
A/duck/Hung/4/75	H4	H4N8	A
A/duck/Hung/8/3/75	H4	H4N8	A
A/duck/Hung/1/75	H10	H10N4	A
A/duck/Hung/11/75	H5	H5N9	A
A/muscovy duck/Hung/1/75	H5	H5N3	A
A/duck/Hung/2/77	H5	H5N2	B
A/duck/Hungary/2/82	H4	H4N6	A
A/duck/Hung/660/87	H10	H10N7	B
A/turkey/Hung/1561/87	?	H9N2	A

- Phylogenetic analysis of the partial HA gene sequences revealed that all Hungarian strains belonged to the Eurasian lineage of AIV. H5 strains were the members of an old European group and the H4 strains originated from three different sources (two from the Far East while one from Europe).
- The genetic analysis of the NA and NS genes showed single or multiple reassortants in the H4 and H5 strains. I have found ten different subtype combinations in 13 independent cases, which suggested that their origin were unlikely the primer reservoir but rather the „artificial" reservoirs of intensive duck farms. This was confirmed by the combination of close HA genes and different NA subtypes (N2, N3 and N9) as well as NS groups found at the H5 subtype.
- The amino acid pattern at the proteolytic cleavage site of the HA confirmed that all H5 and H7 strains belonged to the low pathogenicity (LP) category.

Results with the analysis of the recent strains

- The three waves of highly pathogenic avian influenza (HPAI) H5N1 epidemic affecting Hungary during 2006–2007 were the results of four different introductions of the virus. These are designated groups HU1 – HU4.
- Based on the phylogenetic analysis and the known markers of the genome all strains belonged to clade 2.2 (Qinghai-like viruses) which clade contains furthermore strains from Asia, Europe and Africa. The representative strains of the HU-1 group belonged to sublineage 2.2.B, the HU-2 to sublineage 2.2.1, the HU-3 to 2.2.A1 and the HU-4 to 2.2.A2.
- It was found that the most similar isolates to group HU-1 were found in wild avian species in Croatia and to group HU-2 in Slovakia, Czech Republic and Sweden. It denoted that the swans were infected in a neighbouring area and reached their last station in the latent phase of the infection.
- While group HU-1 and HU-2 viruses persisted for weeks in the same area, reassortation could have happened between the members of the two groups. Sequencing of the whole coding sequence of the two representative strains revealed that each segment clustered with the corresponding clusters indicating the absence of segment reassortment.
- The origin of the geese epidemic remained unclear. The representative strain of the epizootic clustered to sublineage 2.2.A1 forming the HU-3 group (which also includes German French and Swiss isolates from wild birds and geese). Hungarian4 (HUN4) viruses isolated from the third introduction clustered with isolate A/turkey/United Kingdom/750/2007 forming sublineage 2.2.A2, revealing close epidemiological and

phylogenetic relationship.

Publications

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