FIRST CASE OF THE ISOLATION OF THE H3N2 SWINE INFLUENZA VIRUS IN POLAND

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Abstract

The aim of the study was the molecular characterisation of the aetiological agent responsible for the outbreak of swine influenza with mild respiratory tract disorders, which occurred in piglet sector in 2008. RT-nested-PCR for the detection of the conservative region of the matrix 1 gene and subsequently multiplex PCRs were performed to detect HA and NA genes. The amplified mPCR products were sequenced and the phylogenetic alignment was analysed. The new strains isolated from the outbreak were determined as H3N2. They represent the initial newly emerged subtype in our country, and the second one in the Central-Eastern part of Europe. The new Polish isolates were most similar to the new evolutionary SIV strains from Spain, Belgium, and Italy.

Key words: pigs, swine influenza, H3N2 swine influenza virus, Poland.

The surveillance program for swine influenza (SI) was initiated in Poland for the first time at the end of 90s. At the beginning, it was based on serological investigations and virus isolation. Since 2005, molecular techniques have been subsequently implemented, including the detection of the swine influenza virus (SIV) RNA, molecular subtyping, and phylogenetic analysis.

Based on the results of serological examinations, performed over a ten-year period (1998-2008), it may be stated that three subtypes of the swine influenza A virus: H1N1, H1N2, and H3N2, were present in Poland (10). Up to 2007, the dominating prevalence of the H1N1 subtype was evidenced in serological monitoring (14.4% of seroreagents). Moreover, as was estimated in the multiplex PCR tests, clinical disorders connected with SI outbreaks, up to the end of 2007, were caused by the H1N1 subtype only.

The genetic comparison of the sequences of matrix (M) and haemagglutinin (HA) genes showed that most of the Polish SIV isolates, derived from one year of isolation and from the same district of Poland, were 100% similar. The phylogenetic comparisons of the HA gene fragments indicated that they are closely related to the HA genes of the “avian-like” H1N1 viruses, which circulated in Europe. The Polish strains were related mostly to the French and Spanish strains (Cotes d'Armor/1488/99 - 99.5% with Pol/RU1/02; Spain/53207/2004 - 99.5% with Pol/J2/04).

Regardless of an increase in seroprevalence for the H3N2 subtype in large Polish farms, from 2.2% in 2004 to 7.85% in 2007, no H3N2 and H1N2 subtypes were isolated from pigs with the clinical signs in that period. In 2008, a further increase in the percentage of animals possessing the antibodies against H3N2 subtype (to 9.8%) was evidenced. Additionally, at the end of December 2007/beginning of January 2008, a quickly spreading sudden illness with mild respiratory tract disorders occurred in the piglets sector of a farm located in the South-West of Poland. The subclinical course of the disease was observed, including symptoms like mild fever, coughing, nasal serous exudate, slightly laboured respiration, and anorexia. Within the next five days, similar signs occurred in swine in the other sectors. Pleural exudate and diffused inflammatory focuses in the pulmonary lobes, typical for pneumonia, without distinct line demarcation from the healthy tissue, were observed in the euthanised animals. However, the mortality index was zero, and no abortions in the reproduction sector occurred, as described in previous cases of SI in Poland caused by the H1N1 viruses (14), the economic impact of the outbreak was evident. An increase in the number of sows positive in the usg examination, which did not deliver was evidenced. In the three reproduction groups the effectiveness of mating decreased by 10%. In the culmination of the disease, 17 sows had no oestrus symptoms (in that farm, usually four sows showed the aforementioned problem). Additionally, an increase in the mortality by 1% and cachexy signs within 1.5 months from the outbreak’s onset was noted in piglets.

The aim of the study was the molecular characterisation of the aetiological agent responsible for the aforementioned SI outbreak.
Material and Methods

Samples. Ten nasal swabs from the piglets housed in the aforementioned farm and demonstrating influenza-like symptoms, were collected. The swabs were suspended in PBS and 300 µl of each specimen were directly used in the molecular biology testing (RT-nested PCR based on the M1 gene sequence and multiplex PCRs based on the HA and NA genes sequences). Parallely, the swabs were used for the virus isolation in amniotic and allantoic cavities of 10-d-old embryonated hen's eggs. The isolation was performed according to the standard procedure.

Viruses. Three strains of SIV: H1N1 (A/Sw/Bel/1/98), titer EID₅₀ 10⁻⁴/0.2 ml, H1N2 (A/Sw/Eng/96), titer EID₅₀ 10⁻⁷/0.2 ml, and H3N2 (A/Sw/F1/1/98), titer EID₅₀ 10⁻⁵/0.2 ml, were used as reference material.

Nucleotide sequence analysis. Viral RNA was extracted by means of the RNA Total Isolation Kit (A&A Biotechnology, Poland). The reverse transcription was performed by mixing 5 µl of RNA with 15 µl of RT reaction mixture (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl₂, 125 µM each dNTP, 1 unit of RNaze, and 100 units of M-MLV enzyme - Invitrogen). cDNA was synthesised using 50 ng of Random Primers (Invitrogen) in a single reaction.

Subsequently, RT-nested-PCR for detecting the conservative region of the matrix 1 gene was evaluated. Strains with positive results in the first PCR were examined in three multiplex reverse-transcription polymerase chain reactions, carried out to detect the HA and NA genes of the H1N1, H1N2, and H3N2 subtypes. For the three single primer pair and multiplex PCR, each primer set was used at 7.5 pmol. The characterisation of the primer sets used in the examinations is presented in Table 1. The amplicons were visualised by ethidum bromide staining, following electrophoresis on 1.5% agarose gel.

Sequencing and phylogenetic analysis. The amplified mPCR products for the HA genes were sequenced in the GATC-biotech DNA analysis service in Germany. Phylogenetic alignment was performed with representative swine viruses, those whose HA sequences are available in public databases. The nucleotide sequences were compared initially by using the clustal W alignment algorithm method. The phylogenetic tree was determined by the Neighbour-Joining method using MEGA 3 software. The sequences-distance analysis was performed by using the Megalign unit from Lasergene software.

Results

The RNA of SIV was detected in nine out of ten tested specimens. Using the conventional virology method, the SIV was isolated from two samples. The strains isolated from the aforementioned SI outbreak were determined as H3N2 (Fig. 1). This was the first case of the isolation of H3N2 SIV in Poland.

For the phylogenetic analysis, only the HA3 gene sequence was used. The length of the gene region chosen for phylogenetic comparison was 543 bp (position 193-735). The H3N2 isolate was tagged as Sw/Pol/T2/2008 (Fig.2). The set of 56 sequences of the HA gene, available in GeneBank, were included in the analysis. One representative sequence for each geographic region and different year of isolation was chosen. The phylogenetic analysis showed a tree, which included swine, avian, equine, and canine H3 viruses. Sw/Pol/T2/2008 is located in the swine cluster, in the main prevalent European group of H3N2 isolates (Fig. 2).

The main European genetic group is the A/Port Chalmers/1/73-like Eurasian swine H3N2 lineage (known as swine PCh lineage). It evolved separately from the human H3N2 virus lineage around 1973. Within the PCh lineage, the Polish isolate H3N2 is the most similar to a subgroup, which evolved at the beginning of 90s and formed a new antigenic subcluster, with the exception of the USA and Hong Kong SIV isolates from the 70s. Sw/Pol/T2/2008 has the highest similarity with A/Swine/Spain/739139/2002 (97.5%), A/Swine/Belgium/87/95 (96.9%) and A/Swine/Italy/11338/2000 (96.8%). It is the most distanced from the vaccine used in the human population in Europe (A/Port Chalmers/1/73 - 89.3%) and the USA (A/Wisconsin/67e5/2005 - 82.1%).

Table 1

<table>
<thead>
<tr>
<th>Genes</th>
<th>Gene Bank accession number</th>
<th>Primer’s name</th>
<th>Oligonucleotide sequences with nucleotide position according to reference sequence</th>
<th>PCR product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA1</td>
<td>A517815</td>
<td>H1K1F</td>
<td>(71) ACCATGCTAAACAATCCCA</td>
<td>788bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H1K1R</td>
<td>(859) CATCCGACATCATGATCACC</td>
<td></td>
</tr>
<tr>
<td>HA1</td>
<td>AF085416</td>
<td>H12KF</td>
<td>(528) CTTGCGGCTGCAGGGAAGAA</td>
<td>359bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H12KR</td>
<td>(887) TGAGACGATGATTCGTGCC</td>
<td></td>
</tr>
<tr>
<td>HA3</td>
<td>AJ293930</td>
<td>H3-31F</td>
<td>(31) CATTCTATGTCTCGTCTTGG</td>
<td>516bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H3-547R</td>
<td>(547) TATCCGACATCCCGTGG</td>
<td></td>
</tr>
<tr>
<td>NA1</td>
<td>CY010582</td>
<td>N1F</td>
<td>(623) TGGAATACATATGCGCATATATG</td>
<td>512bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N1R</td>
<td>(1135) GAGATCCAAATATCATCTCAA</td>
<td></td>
</tr>
<tr>
<td>NA2</td>
<td>CY010566</td>
<td>N2F</td>
<td>(563) GGAAGAAGCATGGGCTGCAT</td>
<td>790bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N2R</td>
<td>(1353) GTGCACAAACACACAA</td>
<td></td>
</tr>
</tbody>
</table>
Fig. 1. Haemagglutinin and neuraminidase typing by multiplex PCR: M – Low size DNA marker; 1, 2 - tested samples, 3 – (+)ve control; 4 - (-)ve control; 516 bp - PCR product size for HA3, 790 bp PCR product size for NA2.

Fig. 2. Phylogenetic tree for the HA of swine influenza H3N2 viruses and human, avian, and equine reference strains. The H3N2 SIV strain isolated in Poland is marked with a black arrow. The scale bar represents approximately 2% nucleotides changes between close relationships. In the grey box, the SIV known as the PCh-lineage group is located.

Discussion

Swine influenza may have a different clinical picture. Although the severity is affected by many factors, including the viral strain, the onset of the disease is typically sudden and the general signs include anorexia, inactivity, fever, respiratory disorders, coughing, conjunctivitis, nasal discharge, and weight loss (13). Swine influenza is also a herd disease, characterised by high morbidity (approaching 100%) and generally low mortality (1%) rates. In general, the pigs infected with the H3N2 subtype demonstrate clinical symptoms milder than in the case of H1N1 infection. However, the novel H3N2 viruses introduced into North American swine herds in the late 90s induced a dramatically severe disease in the naïve population, which resulted in abortions and an unusually high mortality rate in mature sows (15). The susceptibility of infected pigs to swine influenza virus-induced signs and lesions correlated with the age of the animals and was also dependent on the superior response to the strain, which may highlight the multifactor and multigenic nature of the influenza virus pathogenicity. It is probable that progressive antigenic drift and the shift in the H3N2 genome have an input in the high diversity of the clinical picture and pathogenesis.

In the described case, the occurrence of H3N2 in the pig population caused a low level of morbidity for some part of the herd, and no mortality. It should be emphasised that the described outbreak with the mild subclinical course was noted by the National Veterinary Research Institute for the first time in Poland. It occurred in a year with an increase in the seroprevalence against the H3N2 subtype. As was evidenced in the serological examination, the introduction of an A/Port Chalmers/1/73-like human influenza virus into the pig population occurred for the first time in 1974 at the latest (8). The antigenic characterisation of the swine H3N2 isolates after the 1970s showed that the virus had been derived from its ancestor, and this evolution separated a new cluster. In Europe, porcine H3N2 viruses appeared between 1983 and 1985, which resulted from the reassortment of human PCh-73 H3N1 and avian-like H1N1 (22). So far, the isolation of the SIV H3N2 subtype from pigs was ascertained in Hong Kong in 1968 and 1975, Czechoslovakia in 1975, Italy in the early 1980s, Belgium and France in 1984, Great Britain in 1987, Canada in 1988, and the United States in 1998 (1, 3, 6, 9, 11, 17, 18, 21, 23). In many cases, the H3N2 strain was isolated without any disorders resulting in abortions and an unusually high mortality rate in mature sows (15). The susceptibility of infected pigs to swine influenza virus-induced signs and lesions correlated with the age of the animals and was also dependent on the superior response to the strain, which resulted in abortions and an unusually high mortality rate in mature sows (15). The susceptibility of infected pigs to swine influenza virus-induced signs and lesions correlated with the age of the animals and was also dependent on the superior response to the strain, which may highlight the multifactor and multigenic nature of the influenza virus pathogenicity. It is probable that progressive antigenic drift and the shift in the H3N2 genome have an input in the high diversity of the clinical picture and pathogenesis.

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short average lifespan of the pig in which the immune system of the human counterparts. This is because of the genetic and antigenic changes described in the swine PCh-lineage viruses are approximately slower than the human counterparts. That is because of the short average lifespan of the pig in which the immune pressure may be limited. Furthermore, the vaccination of sows with A/Port Chalmers/1/73-containing vaccines, estimated at about 10% for Northern Europe, could contribute, to a limited extend, to the immunity pressure. The observed antigenic drift of the H3N2 SIV. The genetic and antigenic changes described in the Pch lineage of swine PCh-lineage viruses is approximately slower than the A/Port Chalmers/1/73 strain currently used in commercial vaccines, which could not afford complete protection (7). The results of our phylogenetic analysis clearly demonstrated that viruses isolated from pigs cumulate some mutation, but they do not change quickly. They might represent a different origin and could posses different pathogenesis forces, according to the subtype they represent. Since the time when pigs were first postulated by Brown (2) as being a potential risk in forming a new kind of subtype, they could have been playing an importing role in interspecies transmissions.

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References


