"Diagnostyka szczepów wirusa grypy przy użyciu nowych metod molekularnych."

Abstract in English

It is estimated that each year, seasonal flu epidemics cause from 2 to 5 million cases and 250 000 to 500 000 deaths worldwide. The emergence of a new strain of influenza A virus in 2009 resulted in pandemic that lasted over two years and was responsible for more than 60 million cases in the United States of America alone. These figures show that the newly emerging strains of influenza A virus pose a serious threat to public health.

Lack of resistance to such strains in the human population is caused by continuous genetic variation of the virus, which is based on phenomena such as reassortment (exchange of genetic segments between two different viruses), antigenic shift (change of genetic segments encoding the major surface antigens) or antigenic drift (slow accumulation of small variations in sequences encoding the major surface antigens).

Prophylactic vaccination against currently circulating human strains shows high efficiency, but due to the variability of surface antigens, hemagglutinin (HA) and neuraminidase (NA), it must be repeated every year with the new vaccine, which composition is determined on the basis of antigenic drift. Furthermore, in case of emergence of an entirely new strain, resulting from reassortment, seasonal vaccine will most likely prove ineffective.

Reassortment of genetic segments during coinfection of one organism with strains from different hosts could serve as a potential source of new, dangerous strains of influenza A virus, and may lead to overcoming species barriers and adaptation of the virus to new host. The World Health Organization (WHO), the Food and Agriculture Organization of United Nations (FAO), and the World Organization for Animal Health (OIE) issued many reports about mixed infections between humans and animals, describing the gravity of the problem. All the organizations recommended specific procedures to limit the mixing of different strains of the virus. They also clearly stated that the adaptation of human viruses among farm animals would create a potential for reassortment with other viruses of swine or avian origin and emergence of a novel, more

virulent strain. According to the concept of "One Health", which is now the basis for cooperation between the CDC, WHO, FAO and OIE, human health is related to the animal health and the environment, so we should put great emphasis on monitoring, diagnostics and control of influenza virus infections not only in humans but also in animals remaining in our immediate vicinity, as well as in wild waterfowl serving as the reservoir of all types of the virus, in order to collect data and evaluate potential hazard which may result in emergence of dangerous human and/or animal strain of the virus.

A similar problem is the formation of viral quasi-variants, resulting from erroneous incorporation of the nucleotides by the viral RNA polymerase during replication. Such variants are circulating in a host along with the predominant variant. When the viability of the quasi-variant and dominant variant is similar, the quasi-variant remains in the viral population at a low level. Whereas when the immunological pressure of the host, or environmental pressure, will cause that the quasi-variant will be better adapted to survival in the altered conditions, it may lead to replacement of the dominant. This could lead to evasion of the host immune response by a new variant of the virus (including responses stimulated by vaccinations) and the acquisition of resistance to currently used antiviral drugs.

Most of the techniques used in routine diagnostics failed to detect viral quasi-variants and mixed infection, due to the detection limit and high specificity. Methods based on the RT-PCR and Real-Time PCR techniques, designed to detect a specific sequence, could not reveal the presence of another variant of the virus in the same sample. The accumulation of single mutations resulting in small changes in the genome prevent the probe or primers, used in the above mentioned techniques, from binding to specific region of DNA causing erroneous readings. On the other hand, serological tests and immunoassays do not have sufficient sensitivity to distinguish between two variants of the virus. The new methods of sequencing and mass spectrometry, which currently are used to determine the level of prevalence of mixed infections and quasi-variants, may be the answer to this problem, but because of the costs and time-consuming preparatory procedures their use in routine diagnosis is in question. Bearing in mind these limitations, a method developed during my PhD studies can serve as a useful alternative for the aforementioned diagnostic techniques. This method is based on Multitemperature Single Strand Conformational Polymorphism (MSSCP) and due to its

high sensitivity and low level of detection, it works well in detecting mutations, mixed infection and minority variants of the virus.

MSSCP method is an extension of Single Strand Conformational Polymorphism (SSCP) technique first described in Orita et al. 1989. In a standard technique single-stranded DNA are separated on a polyacrylamide gel, during the separation different three-dimensional structures are formed, with different mobility, depending on the sequence, ionic strength, pH and temperature. In the MSSCP method, electrophoretic separation is carried out in the gel subjected to strictly controlled temperature changes, which increases the likelihood that analyzed PCR product adopt different conformations depending on changes in the sequence, which provide a higher detection sensitivity at the shorter time of analysis.

In the attached publications I have proposed the use of a method based on the MSSCP technique, to:

• Detection and differentiation of mixed infections with seasonal and pandemic strains of influenza A virus among humans

• Studies of genetic variation of the hemagglutinin gene from the A(H1N1)pdm09 pandemic strain and its potential impact on the regions responsible for the interaction with the neutralizing antibodies

• Analysis of co-infections with different variants of influenza A/H1N1 strain among the population of pigs

In the first publication, samples taken from 23 patients showing flu-like symptoms, from various hospitals across Poland, who had been diagnosed earlier with pandemic A(H1N1)pdm09 virus using real time RT-PCR, were analyzed using MSSCP based method. Analysis confirmed the presence of influenza A(H1N1)pdm09 in all the samples. Moreover, in 4 out of 23 samples, MSSCP analysis showed a coinfection with seasonal and pandemic variant of influenza A/H1N1 strain. Direct sequencing of analyzed genetic material confirmed obtained results. Described method allowed not only to specifically differentiate the particular influenza A virus strains, but also detection of minor genetic variant in co-infected sample, with high sensitivity.

The second publication describes the MSSCP analysis of a specific region of the hemagglutinin gene, comprising the sequence of one of the major epitopes for neutralizing antibodies, in isolates of pandemic influenza A(H1N1)pdm09 strain obtained from patients in Taiwan in 2009-2011. The analysis revealed changes in the

nucleotide sequence of isolates obtained between 2010 and 2011 compared to reference pandemic strain from 2009. Most of the changes did not result in serious modifications of the protein structure, but three of them were located within the E epitope, and one of them (E66K) was located in the site responsible for binding of neutralizing antibodies, which could affect the effectiveness of the vaccine. Thus, the studies indicate the utility of the described method for analyzing the genetic variation of specific regions of the genome of influenza A virus and detecting the potential viral *quasi*-variants that may evade the host immune response.

In the third publication, samples subjected to MSSCP analysis were obtained from pigs with influenza-like symptoms isolated in a few farms in Poland. All isolates were characterized earlier as swine "avian-like" A/H1N1 using RT-PCR techniques. The MSSCP analysis revealed the presence of several genetic variants of influenza A virus in tested samples, which shows that the described method can be used for the detection of mixed infection with influenza A virus and the differentiation of specific variants of the virus circulating in the population of pigs.

The fourth publication is a review article describing the usefulness of methods based on the MSSCP technique for genotyping isolates of influenza A virus and other specific applications of this technique. This work describes both the method developed by me during my doctorate, as well as other methods based on MSSCP technique for analyzing genetic variation of viruses.

All listed publications, demonstrate the usefulness of the method which I have developed, for differentiating and detecting specific variants of influenza A virus, for the detection of co-infections of the different variants of influenza A virus in one sample, and analysis of the genetic variation of specific regions of the genome of influenza A virus.