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Influenza diagnosis and vaccination in Poland

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A B S T R A C T

In Poland between several thousand and several million cases of influenza and suspected influenza cases are registered, depending on the epidemic season. A variety of methods are available for the detection of the influenza viruses responsible for respiratory infection starting with the isolation of the virus in chick embryos or in cell lines such as MDCK, VERO, etc., and finishing with a variety of modifications of the classical PCR molecular biology such as PCR multiplex and Real-Time. The most effective way to combat influenza is through vaccination. Regular vaccination is one of the few steps that may be taken to protect individuals, especially in high-risk groups, from the potential and serious complications of influenza. In many countries, including Poland, despite the recommendations, the rate of vaccination against influenza is still low in all age groups. In the epidemic season 2011/2012, the level of distribution of the seasonal influenza vaccines was 4.5% of the population.

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1. Introduction

The statement made by the American researcher Kelvin Sullivan in the last century that influenza is the ‘last uncontrolled plague of mankind’ continues to be valid at the beginning of the twenty-first century. Influenza is not a disease with pathognomonic characteristics and therefore the diagnosis of the infection based upon clinical symptoms is only possible during an epidemic. It is known that influenza-like symptoms may occur in infection with more than 200 viruses including parainfluenza virus, adenovirus, rhinovirus, coronavirus, respiratory syncytial virus, and Coxsackie virus which cause disease and are prevalent at the same time as the influenza virus (Brydak, 2008). It is important to bear in mind that material sampled from the upper respiratory tract can be examined in a variety of ways. Therefore, the laboratory confirmation of infection with influenza virus is important in the control of influenza, and it plays an important role in the evaluation of the effectiveness of vaccines and anti-influenza medication (Landry, 2011). The basic virological laboratory diagnosis of influenza involves confirmation of the presence of viral antigen in specimens from the patient and serological confirmation of influenza virus infection based on an increase in the level of antibodies detected in the patient’s serum (Brydak, 2008, Landry, 2011).

Influenza is a disease in which the continuous evolution of the virus is essential for the occurrence of annual epidemics in the human population, and of an occasional pandemic. Complications from influenza can occur including: respiratory and other systems such as the circulatory system, causing graft rejection, otitis media, myocarditis and pericarditis, toxic shock syndrome, myositis, and myoglobinuria, which may lead to renal failure, glomerulonephritis, exacerbation of chronic glomerulonephritis, worsening of chronic kidney failure, neurological complications, central nervous system, cerebrovascular disease, encephalitis, complications in women who are pregnant, etc. (Brydak, 2008).

Although infection with influenza virus causes multi-organ complications and often deaths in all age groups, ranging from 500,000 to 1 million individuals worldwide, the proportion of the population vaccinated in Poland places the country at the bottom of the list for Europe (Brydak, 2008). This begs the question: will our views on prevention through vaccination only change with the death of a loved one?

The aim of this article is to review the current molecular methods of influenza diagnosis and prevention; the latter, namely through vaccination.

2. Methods of influenza virus culture

Since 1935, isolation of influenza virus grown in chick embryos has become the gold standard for the diagnosis of influenza. This must be performed despite being laborious, often taking up to a
dozen or so days. Patient specimens often require several passages to obtain a viral hemagglutinin titer, which can then be used for a variety of tests (Krauss et al., 2012). Isolation of the influenza virus responsible for the current infections, its identification, and the determination of the antigenic similarities between the strains circulating during a given season are necessary for the development of vaccine, i.e., for the selection of the candidate strains for the vaccine. These steps also are important from the epidemiological standpoint (George, 2012).

For many years isolation of the influenza virus has been performed in both chick embryos and in tissue culture. It was known for some time now that propagation in tissue culture has not been possible in some cases, although other methods have confirmed the presence of influenza virus in specimens from the patients concerned. Therefore, in the first instance replication of the virus should occur in chick embryos. It is worth noting that influenza vaccine is currently also produced in tissue culture, such as MDCK and Vero (Dwyer et al., 2006).

The early, correct and complete diagnosis of influenza is of particular importance at this time, not only in terms of treatment, but also to detect new unidentified pathogens, as was the case many times in the past in 1997, 1999, or 2009 (Brydak, 2008; Ganzenmueller et al., 2010). Isolation of influenza virus from a patient gives valuable information about the type and subtype of virus circulating in the territory of the given population which is very important in the context of the Global Influenza Surveillance Response System (Brydak, 2008). It is also pertinent to the costs incurred for the treatment of patients and their hospitalization in the case of misdiagnosis (Nitsch-Osuch et al., 2012).

3. Methods for detection of influenza virus antigens

Traditional diagnostic methods for the detection of influenza virus in specimens from patients also include analysis based on the detection of viral antigens in immunofluorescence, immunoenzymatic, and immunochromatographic assays (Brydak, 2008). Serological methods are much faster and less costly than viral culture methods, but may have a lower sensitivity and specificity compared with the culture methods described above. Immunofluorescence tests (IF), which enable the identification of antigens of a specific type of influenza virus in clinical samples are used frequently (Kapeluszniak et al., 2009; Berg et al., 2011:). The method utilizes specific antibody labeled with a fluorescent dye such as fluorescein which binds to a particular antigen of influenza virus type A or B. A positive result is obtained when the antigen–antibody complex emits a green light visible against the contrasting background stain for human epithelial cells. Readings are taken with a fluorescent microscope (Nutter et al., 2012). Direct fluorescent–antibody testing has a high specificity (99%), but lower sensitivity (65%) (Bakerman et al., 2011).

Immunoenzymatic tests (IE) are also used in diagnostic laboratories for the rapid detection of influenza virus antigens. Several evaluations comparing immunoassay with the classical method of virus culture have shown that the sensitivity of IE is highly variable and ranges between 44–95% for both types of influenza virus (Jennings et al., 1981; Rapicetta et al., 1982). From December to March during periods of greater incidence of respiratory infections, an increase in IE influenza virus sensitivity to 74–100% is also evident (Petric et al., 2006). This relationship can be accounted for by the decrease in human immunity typical of the autumn–spring season, which may contribute to the more rapid replication of influenza virus and the relatively long persistence of viral particles in the body (Brydak, 2008; Kumar and Henrickson, 2012). IE tests enable the simultaneous detection and differentiation of influenza A and B. On the basis of tests performed on nasopharyngeal aspirates from patients, the specificity of the test for influenza A and B was 100%, while the average sensitivity for influenza type A was 82.9% and for influenza B was 51.5% (Brydak, 2008, 2012).

Rapid tests for the detection of influenza virus antigen, based on the method of immunochromatography (IC) are also available. These tests provide confirmation of the presence of influenza viruses within 15 min (Nitsch–Osuch et al., 2012). Since they are simple to use, they find practical application especially in the setting of primary care and family medicine (Nitsch–Osuch et al., 2013). An evaluation of the diagnostic value of rapid immunochromatographic tests for the detection of antigen influenza virus type A and B in specimens from the respiratory tract revealed that the sensitivity and specificity of detection of the two types of virus was 65% and 100% respectively (Kok et al., 2010; Nitsch–Osuch et al., 2012). It should be stressed that the diagnostic sensitivity of different types of strips, cassette immunochromatographic tests and immunoassays is not very much less reliable than for reverse transcriptase polymerase chain reaction (RT–PCR). However, they are easier to perform and do not require the use of equipment or highly qualified personnel (Petrozzino et al., 2010; Takahashi et al., 2010). Furthermore, the estimated cost of a single IC test is about 10 times lower than the costs incurred in the case of molecular methods. These tests are generally designed for use in the acute phase of infection, when virion numbers are not less than 10^5–10^6 PFU (plaque forming unit). Samples taken too late in the recovery period or stored improperly may contain antigen concentrations below the detection limit (Brydak, 2008; Jacobs and Raja, 2012; Nitsch–Osuch et al., 2012). Any doubts about the reliability of the result, especially when it is inconsistent with clinical signs, should be resolved on the basis of the reference test applying molecular biology techniques (Biggs et al., 2010). In the case of influenza virus this is the RT–PCR method using specific primers and the corresponding thermal profile of the amplification reaction (George, 2012).

Virological diagnosis also includes serological tests, involving the detection of specific antibodies against a defined type of influenza virus, although they are of limited use especially in the rapid diagnosis of respiratory infections (Brydak, 2008). When employing such methods, particular attention should be given to the time elapsed since the onset of symptoms. This type of test involves a measurement of the level of specific anti-influenza antibodies most often that of antihemagglutinin (Brydak, 2012). Changes in the level of antibodies between serum samples from the period of acute illness and of that of recovery or after a few weeks may confirm or refute the presence of infection with influenza virus. The hemagglutination inhibition test (HI) and neuraminidase inhibition test (NI) are performed very frequently in research studies to evaluate the humoral immune response after vaccination against influenza virus (Brydak, 2008).

4. Virological methods based on molecular biology

Nucleic acid amplification methods play an increasingly important role in the diagnosis of influenza virus. They are characterized by much higher sensitivity and specificity than other classical techniques. The molecular detection of viral RNA also provides for the early detection of influenza virus infection, already at the onset of symptoms, before the virion numbers reach the level required by other methods (Brydak, 2008; Landry, 2011). The polymerase chain reaction (PCR) is a highly specific amplification of the selected nucleic acid fragment, specific to the type of influenza virus and is determined by the sequence of the specific primers used in the reaction (Poon et al., 2009). Visualization of the resulting PCR amplification products is made possible by their separation in agarose gel (less frequently in polyacrylamide gel) and the use of
fluorescent dye, e.g., ethidium bromide which intercalates into the double-stranded DNA (Brydak, 2008). Influenza virus belongs to the Orthomyxoviridae family which store their genetic information in the RNA sequence. The identification of influenza virus requires recoding as a DNA sequence (derived complementary DNA-cDNA) before amplification of the characteristic fragments of its genome. The modification of PCR by adding a reverse transcription step is known as reverse transcriptase-PCR. The RT-PCR reaction may take two forms: a one stage process (one-step RT-PCR), where both the reverse transcription and PCR reactions are performed in one test tube; alternatively, a two stage process (two-step RT-PCR), with transcription of viral RNA into cDNA in the first stage and amplification of certain sequences in the second stage (Brydak, 2008, 2012). Numerous scientific reports indicate that the two-step process is characterized by better sensitivity (Poon et al., 2009; Wang and Taubenberger, 2010). In order to detect the presence of factors in the test sample which inhibit the PCR reaction, internal control is performed using primers which are complementary to the sequence of the reference gene, and which are always expressed in human epithelial cells. These genes include β2-microglobulin, β-actin, γ-actin, RNA-ase and ornithine decarboxylase (Brydak, 2012). In view of the large variability of influenza virus its detection in a clinical specimen necessitates the use of primers which are complementary to highly conserved gene sequences and regions with a minimal variation within these genes. The most conserved influenza virus genes are those encoding the M protein and the nucleoprotein, and usually they are used to design primers/probes. In order to differentiate strains of influenza virus type A, primers/probes are hybridized to conserved regions in the gene for hemagglutinin or neuraminidase (Brydak, 2008). Conventional PCR methods are increasingly being replaced by real-time PCR, using fluorescent techniques, which enables monitoring of the amplification product in each cycle of the PCR reaction (Brydak, 2008). It is preferred because of some simplification of the automation technology and of a significantly less time-consuming process. The main advantage of this method is its quantitative nature, in contrast to the classical PCR method which provides a qualitative result (present/absent) only. Labeled probes bind to the replicated viral RNA providing for the measurement of the number of particles which allows for the determination of the initial level of viremia and for monitoring of the effects of therapy in patients with influenza (Brydak, 2012). The main disadvantage of this diagnostic method is its cost. Scientific reports indicate that test duration much shorter than 24 h and the rapid confirmation of the viral etiology of infection may explain the use of such an expensive test for routine diagnostics (George, 2012; Wang and Taubenberger, 2010). Furthermore, refraining from administering antibiotics and the rapid inclusion of appropriate treatment are factors which support the use of this method. A test has been developed based on the method of multiplex real-time PCR, which makes possible the simultaneous identification of influenza virus type A and B, parainfluenza 1–3, hMPV, RSV, rhinovirus, enterovirus, adenovirus, coronavirus (229E, OC43, NL63) and the atypical Mycoplasma pneumoniae and Chlamydia pneumoniae microorganisms in nasopharyngeal secretions. The authors of the report on this matter estimate that the cost of the analysis of a single clinical specimen in this test is significantly lower when compared to the standard methods of detecting single pathogens and amounts to about 33 Euro (Brydak, 2008).

5. Influenza vaccines in Poland

Since 1941, it is known that influenza vaccination is the cheapest, most effective way to fight this infection (Brydak, 2008). Currently, medical practitioners have available on the market many types of inactivated influenza vaccines, with different variants containing different adjuvant or without it (Brydak, 2008, 2012). Another type of vaccine is derived from the so-called cold adapted influenza virus that is adapted to a reduced replication temperature (Brydak, 2008). Both these technologies allow for the development of the vaccine in egg or MDCK and vero tissue culture. Methods to produce clean, safe, and effective inactivated vaccine have been applied for many years. Currently, the use of the latest molecular biology techniques means that strains of influenza virus used for vaccination against influenza, appear to be almost 100% compatible with those that appear in the following epidemic season. Influenza vaccines prepared in egg or tissue culture are registered in Poland and listed in Table 1 (Brydak, 2012).

6. Influenza vaccine coverage in Poland

Table 1 shows the inactivated influenza vaccines registered in Poland (Brydak, 2012). The Advisory Committee on Immunization Practices (ACIP) first recommended seasonal influenza vaccination for all persons aged ≥ 6 months. Vaccination should be offered throughout the influenza epidemic season. As recommended by the ACIP (2011a,b) and 14 other international scientific societies, it is essential to vaccinate the greatest possible percentage of the population in the world (Brydak, 2008, 2012). Those vaccinated against influenza should include individuals with clinical indications, i.e., high-risk groups, particularly vulnerable to the occurrence of complications as well as those healthy individuals in a position to transmit influenza to persons at high risk and who can be identified as a source of infection for these people. According to data from the National Institute of Public Health, National Institute of Hygiene (NIPH-NIH) in 2011, the proportion of the population vaccinated against influenza per age category in years remains at a very low level. The level of vaccination of children from 6 months to 4 years of age is shamefully low at only 0.85%. Vaccination levels are somewhat better for children aged 5–14 at 1.49% and those aged 15–64 at 1.95%. The percentage of the vaccinated population ≥ 65 years according to NIPH-NIH data is not satisfactory at a level of 8.46%. Even though the Marshal Offices of the leading Provinces have offered free influenza vaccination to individuals aged ≥ 65 years for a few epidemic seasons the percentage of the vaccinated population in this age range is very low fluctuating around 30% (Brydak, 2012). Embarrassingly by contrast, vaccination remains at a low level 6.4% for health workers. In the 2011/2012 season, only 4.5% of the population in Poland was vaccinated, despite the

| Table 1 | Influenza vaccines registered in Poland in 2011 (Brydak, 2012). |
|---------|---------------------------------------------------------------|
| **Influenza vaccine received in cultured chick embryos** | Inactivated influenza vaccine split virion |
| VAXIGRIP (Sanofi Pasteur SA, F) | Fluvarix (GlaxoSmithKline, B) |
| Begrivac (Novartis Vaccines and Diagnostics, D) | | **Influenza vaccine received in the MDCK cell culture** |
| ID Flu (Sanofi Pasteur SA, F) | Inactivated subunit influenza vaccine, containing isolated surface antigens, hemagglutinin and neuraminidase |
| Subunit vaccines containing isolated surface antigens, hemagglutinin and neuraminidase | Influvac (Abbott, NL) |
| Agrippal (Novartis Vaccines and Diagnostics SRL, I) | **Influenza vaccine received in the vero cell culture** |
| Virasomal vaccines | Inactivated influenza vaccine with split virion |
| Inflexal V (Berna Biotech I, S.L) | Preflucl (Baxter, A) |

* Vaccine administered intradermally.
fact that the ternary composition included the pandemic virus (Brydak, 2008). Possible strategies to increase influenza vaccine coverage include: implementation of national influenza immunization programme, personal invitation letters or telephone calls for patients, effective communication about performance and methods used to identify and contact eligible patients, educational activities dedicated both for patients and medical professionals, providing patients with free of charge vaccines (reimbursement) (Brydak, 2008).

In terms of the percentage of the population vaccinated against influenza, Poland holds the penultimate place in Europe (Brydak, 2008). Reasons for the low prevalence of influenza vaccination include: lack of sufficient acceptance of this form of prevention by doctors, lack of awareness about the legal obligation of doctors to recommend vaccination, insufficient knowledge about the cost of influenza and that of its complications, not only in individual cases, but for the country as a whole, insufficient knowledge about the types of vaccines, the expectation that vaccination protects against all upper respiratory tract infections, confusion of influenza with the common cold example, so called stomach flu or other respiratory infections, frequent adverse events after vaccination and confusion with the complications of influenza, the need to repeat vaccination every epidemiologic season (Brydak, 2008, 2012). There are also many myths within society that must be shown to be false such as the idea that inoculation causes infection. In Poland, the influenza vaccines are inactivated and they contain fragments of killed virus: split and subunit, which is unable to multiply in the body and to cause disease. Serious problems (including anaphylactic reactions) from inactivated influenza vaccine are very rare. Some minor side effects that could occur are: local side effects (soreness, redness, or swelling where the shot was given), fever (low grade), aches, headache, fatigue (Brydak, 2008). One should be aware that apart from the recommendations of ACIP (2011a, b), also 14 other scientific societies, listed in Table 2, also recommend vaccination against influenza. Increasingly, the issue of vaccination is considered not only from its health aspects but also in terms of economic impact, which is not often taken into account.

Studies conducted in the Influenza Viruses Research Department of the National Influenza Center NIPH-NIH in 1993/94–2006/07 in collaboration with clinicians, including vulnerable groups, clearly indicate the possibility of producing a humoral immune response in these patients, and the consequences of protecting them against the harmful complications of influenza, as shown in Table 3. The studies aimed to convince not only those at high risk, but also medical personnel about the necessity of influenza vaccination, especially in this group.

### Table 2

| Advisory Committee on Immunization (ACIP) |
| American Academy of Pediatrics (AAP) |
| American Academy of Family Practice (AAFP) |
| American Academy of Family Physicians (AAFP) |
| Working Party on Preventive Services (USPSTF US) |
| American College of Internal Medicine |
| American Society of Internal Medicine, US |
| American Society for Infectious Diseases (IDSA) |
| Canadian Working Group on Periodic Health Research |
| American Cancer Society |
| American College of Obstetrics and Gynecology |
| Executive Director of the Department of Health UK |
| American Heart Association/American College of Cardiology |
| Global Initiative on Chronic Obstructive Lung Disease (GOLD) |
| Global Strategy for Recognition, Treatment and Prevention of Asthma (GINA guidelines) |

### Table 3

| Studies conducted in the Research Department of Influenza Viruses, the National Influenza Center, NIPH-NIH in Poland in collaboration with clinicians in the groups at risk and evaluated the humoral immune response to influenza vaccination (Brydak, 2008, 2012). |
| Children |
| Children aged 6–35 months, 3–8 years, 9–12 years, 0.13–20 years (Brydak, 2004) |
| Children with acute lymphoblastic leukemia (ALL), vaccinated at different times after treatment (Brydak et al., 1998a, b, 1997c) |
| Children with severe hemophilia (Brydak et al., 1998a, b) |
| Children with bronchopulmonary dysplasia (Brydak et al., 1997b) |
| Children with glomerulonephritis (Brydak et al., 2000) |
| Children with chronic renal failure subjected to continuous ambulatory peritoneal dialysis, hemodialysis, and chronic renal failure vaccinated once and twice (Brydak et al., 2001b) |
| Children infected with HIV (Brydak et al., 1999) |
| Children vaccinated after splenectomy in age groups 0-5 years, 6-10 years, 11-15 years (Brydak et al., 2004a, b) |
| Children with aplastic anemia, children with bronchial asthma, children with inflammatory bowel disease (Brydak, 2008) |
| Adults |
| Adults aged 21–30, 31–40, 41–50, 51–64, >64 years (Brydak et al., 2003, 2004b) |
| Billeted students of the Military Medical Academy (Brydak et al., 1997a) |
| Patients with acute lymphoblastic leukemia (Brydak and Calbecka, 1999) |
| Patients with chronic renal failure (Kozioł-Montewka et al., 1997) |
| Patients after renal allotransplant recipients (Wyzgal et al., 2002) |
| Patients infected with HIV at various levels of CD4, with symptoms of AIDS and asymptomatic (Brydak et al., 1999) |
| Patients with breast cancer (Brydak et al., 2001a) |
| Patients with asthma (Jahn–Rozyk et al., 2004) |
| Patients with a group of young and elderly (Brydak et al., 2003) |
| Patients with acute cardiovascular events (Cziesowski et al., 2008) |
| Patients with malignant lymphomas (Brydak et al, 2005) |
| Patients with lupus (Wiesik-Szewczyk et al., 2010) |
| Patients with primary systemic vascular inflammation: Wegener’s granulomatosis (Życzinska et al., 2007) |

### 7. General recommendations for physicians regarding seasonal influenza vaccination

Regular vaccination should be part of sound medical practice and our own health care and that of our family. Therefore, it is recommended that they undergo seasonal vaccination as a protective measure (Brydak, 2008, 2012). On the basis of very extensive literature it is clear that vaccination against influenza prevents many complications, mortality, and also brings measurable economic benefits to the whole state budget. It is noteworthy that complications from influenza infection may occur in previously healthy individuals. According to Article 51 of the Act of 5 December 2008, on the prevention and fight against infections and infectious diseases in humans (Journal of Laws 2008 No. 234, item 1570) which entered into force on 1 January 2009, the physician must inform the patient about the provisions of the Act concerning protective vaccinations listed in the relevant ordinances. Failure to fulfill this requirement may result in criminal prosecution (judgment according to the provisions of the Act dated August 24, 2001 – Code of Petty Offenses Acts (Journal of Laws 2008 No. 133 and No. 214 item 848, item 1344). The offering and organizing of vaccination against influenza, especially for those in high-risk groups, is regarded as an ethical obligation.

### 8. Conclusions

Effective detection of respiratory viruses including influenza virus is possible thanks to advances made in recent years in the
diagnosis of virological infections. Selection of appropriate tests depends on the type of virus to be detected and presumed amount of antigen, the population of patients and the technical capabilities and experience of analysts. An important clinical aspect is the selection of tests that provide information in less than 24 h. In the diagnosis of influenza the methods in most common use include immunofluorescence tests, due mainly to the short duration and relatively simple methodology and molecular tests, using nucleic acid amplification, not only because of the high sensitivity and specificity, but also because of the concurrent detection of other pathogens from the same clinical specimen. In order to develop the most appropriate vaccines for each epidemic season, the 139 National Influenza Centers currently operating throughout the world send one thousand isolates to the WHO Reference Centers for Influenza and the application of molecular diagnostics provides for typing and subtyping of the influenza virus and for the determination of the appropriate composition of the vaccine for a given the epidemic season.

Conflicts of interest

The authors declare no conflicts of interest in relation to this article.

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