© Pediatr Med Rodz 2018, 14 (3), p. 286–292
DOI: 10.15557/PiMR.2018.0032

Received: 12.06.2018
Accepted: 26.06.2018
Published: 31.10.2018

Sainjargal Byambasuren[1], Lidia B. Brydak[2]

Laboratory diagnosis of influenza

Diagnostyka laboratoryjna grypy

[1] "EASTMED" Family Medicine, Acupuncture, Aesthetic Medicine, Warsaw, Poland
[2] Department of Influenza Research, National Influenza Centre, National Institute of Public Health – National Institute of Hygiene, Warsaw, Poland
Correspondence: Lidia B. Brydak, Chocimska 24, 00-791 Warsaw, Poland, tel.: +48 691 352 979, e-mail: brydaklidia@gmail.com

Abstract

Influenza has always been and still is the cause of considerable morbidity and, consequently, frequent multiorgan complications, often irreversible and even fatal. It is an acute infectious disease caused by type A, B and C viruses, members of the family *Orthomyxoviridae*. Infections caused by the influenza virus are reported in every epidemic season. Influenza infections should be considered not only in the aspect of health, but also in the quantifiable, measurable economic aspect. For many years, influenza has been one of the basic priorities of public health. Virological and epidemiological surveillance of influenza, which is implemented in each epidemiological season, is one of the key elements of public health. Virological surveillance involves laboratory confirmation of infection, while epidemiological surveillance involves monitoring of actual and suspected cases of influenza. Laboratory diagnosis is performed to confirm influenza virus antigen in the material collected from the patient, isolate the virus and confirm viral infection based on increased serum antibody levels. Isolating influenza viruses that circulate in a given epidemiological season is necessary to prepare a vaccine against influenza. An early and correct virological diagnosis of respiratory infection, with particular reference to influenza, is currently of great importance in terms of both medical and economic aspects. The paper discusses influenza diagnostic methods currently used in Poland to help physicians in deciding whether laboratory confirmation of diagnosis is justified in the aspect of possible treatment to avoid influenza-induced multiple organ complications.

Keywords: influenza, diagnostic test, molecular biology methods, serological methods

Streszczenie

Grypa była i jest przyczyną licznych zachorowań, a w konsekwencji niejednokrotnie wielonarządowych powikłań pogrypowych, często nieodwracalnych komplikacji prowadzących do zgonu. To ostra choroba zakaźna wywoływana przez wirus grypy typu A, B, C należący do rodziny *Orthomyxoviridae*. Zakażenia wywoływane przez wirus grypy rejestrowane są w każdym sezonie epidemicznym. Infekcje grypowe należy rozpatrywać nie tylko w aspekcie zdrowotnym, ale również policzalnym, wymiernym aspekcie ekonomicznym. Grypa od wielu lat należy do podstawowych priorytetów zdrowia publicznego. Jednym z istotnych elementów zdrowia publicznego jest wirusologiczny i epidemiologiczny nadzór nad grypą, prowadzony w każdym sezonie epidemicznym. Nadzór wirusologiczny obejmuje laboratoryjne potwierdzenia zakażenia, natomiast nadzór epidemiologiczny to monitoring przypadków zachorowań i podejrzeń zachorowań na grypę. Diagnostyka laboratoryjna grypy polega na potwierdzeniu antygenu wirusa grypy w materiale pobranym od chorego, wyizolowaniu wirusa grypy oraz potwierdzeniu zakażenia wirusem grypy na podstawie wykrycia przyrostu poziomu przeciwciał w surowicy. Wyizolowanie krążących wirusów grypy w danym sezonie epidemicznym jest niezbędne w celu przygotowania szczepionki przeciwko grypie. Przeprowadzenie możliwie wcześnie prawidłowej diagnostyki wirusologicznej infekcji układu oddechowego, ze szczególnym uwzględnieniem grypy, ma bardzo duże znaczenie, zwłaszcza obecnie, zarówno pod względem leczniczym, jak i ekonomicznym. W niniejszym artykule przedstawione zostały aktualnie stosowane w Polsce metody diagnostyki grypy – zawarte w nim informacje mają pomóc lekarzom w podjęciu decyzji o zasadności laboratoryjnego potwierdzenia diagnozy w aspekcie możliwości leczenia w celu uniknięcia wielonarządowych powikłań pogrypowych.

Słowa kluczowe: grypa, badanie diagnostyczne, metody biologii molekularnej, metody serologiczne

Influenza is an acute infectious disease caused by type A, B and C viruses of the family *Orthomyxoviridae*. The virus is transmitted by inhaling microscopic respiratory secretions while in close contact with the patient; with the highest infectivity during the asymptomatic period[1]. According to World Health Organization data, the estimated annual attack rates are 5–10% in adults and 20–30% in children, with severe illness reports in 3–5 million cases, including about 250 000–635 000 deaths[2,3]. It is important to realise that influenza is a seasonal disease, but due to the registration of infectious and non-infectious diseases as well as the finances of the state budget, the data are given annually.

The clinical picture of influenza is characterised by sudden-onset and high infectiousness. The incubation period is followed by:

- **general symptoms** – malaise, general fatigue, chills, hyperaesthesia, fever above 38°C;
- **respiratory symptoms** – serous nasal discharge, sore throat, hoarseness, chest pain, dry cough provoking vomiting;
- **symptoms from other systems** – headache, lack of appetite, muscle pain, dizziness, diarrhea, abdominal pain, nausea and vomiting. Somnolence is observed in about 50% of children under 4 years of age and only 10% of children aged between 5 and 14 years of age. Gastrointestinal symptoms, nausea and vomiting in particular, are very common in children and less common in adults[4,5]. The clinical course of viral disease depends on the type of virus, patient's age, immune status, tobacco smoking, comorbidities, such as heart or lung diseases, renal function, immunosuppression, pregnancy, nutritional status and adherence to basic hygiene principles[5]. Influenza virus occurs every epidemiological season and is characterised by high infectivity, possible infection and death regardless of patient's age, constant mutations, influenza-induced complications, measurable economic consequences, and absolute necessity of preventive actions[2,4–8].

Due to the similarity of the clinical picture and the course of the disease to the so-called influenza-like diseases, the diagnosis of influenza based on the clinical symptoms is possible only during epidemics. In viral respiratory infections, the same virus may cause different clinical manifestations, but at the same time, the same clinical symptoms may be caused by different viruses. Therefore, material collected from the upper respiratory tract is likely to be analysed in a multidirectional way[8–10]. Various commercial sets using molecular biology methods have been available for many years to detect, for example, 15 respiratory pathogens. These include type A and B influenza virus, type A and B respiratory syncytial virus (RSV), parainfluenza virus types 1–4, human metapneumovirus (hMPV), adenoviruses, rhinoviruses, coronaviruses 229/NL63 and OC43/HKU1 and enterovirus[9,10]. The above-mentioned test as well as other virological assays using molecular biology, e.g. the subtype or the line of type B influenza virus, may be performed in the Department of Influenza Research, National Influenza Centre at the National Institute of Public Health – National Institute of Hygiene in Warsaw. Depending on the type of test, the results are obtained within several hours[9,10]. This is of key importance for the initiation of treatment, which should be implemented 36–48 hours after the onset of first symptoms and involve the use of new generation anti-influenza drugs[5,8,11–15].

Influenza is not a pathognomonic disease. Influenza-like symptoms may be caused by more than 200 viruses, including parainfluenza, adenovirus, rhinovirus, coronavirus, RSV, *Coxsackie* viruses and hMPV causing disease at the same time as the influenza virus. For this reason, laboratory confirmation of influenza virus infection is essential for the control of influenza, as well as is an important element of both public health, and the assessment of the effectiveness of vaccines and new-generation anti-influenza drugs[8,12,15].

Although the symptoms of influenza are quite characteristic, they cannot be the only basis for establishing a reliable and complete diagnosis, especially in the interepidemic periods. Therefore, it seems necessary to perform diagnostic laboratory tests, including virology and serology, both of which are of particular importance in patients whose immune system is not fully functional, i.e. small children, chronically ill patients, patients on immunosuppressive therapy, the elderly and high-risk patients, who may experience severe illness and complications compared to potentially healthy individuals (high risk of complications)[1,4,8,12]. For this reason, it is important to rapidly identify the aetiological factor to decide on further management, considering the possible use of new generation anti-influenza drugs – neuraminidase inhibitors[4,7,8,11,13].

Laboratory diagnosis involves:

- confirmation of influenza virus antigen in the material collected from the patient;
- serological confirmation of influenza virus infection based on increased serum antibody levels.

The following methods may be used to confirm the presence of influenza virus:

- detection of influenza virus-specific RNA based on RT-PCR (different molecular biology methods);
- immunofluorescence (IF);
- enzyme-linked immunosorbent assay (ELISA);
- bedside tests;
- influenza virus isolation:
  - in 11-day-old chick embryos,
  - in tissue cultures, e.g. MDCK, MDCK-SIAT 1.

VIROLOGICAL DIAGNOSIS

Molecular diagnosis is one of the most dynamically developing fields of biology and medicine. The main differences between conventional and molecular diagnostics include shorter duration as well as increased specificity and

 DOI: 10.15557/PiMR.2018.0032

sensitivity; therefore, molecular diagnostics has become increasingly used in different fields of science, including biotechnology and medicine[5,7,9,11].

For many years, various molecular biology techniques have aroused the greatest interest among the methods of virological diagnosis. These methods, e.g. polymerase chain reaction (PCR), reverse transcriptase PCR (RT-PCR) and real-time or quantitative PCR (qPCR) allow for the detection of viral genetic material in a sample even at low viral levels in the material collected from the patient. Furthermore, the material does not have to be freshly collected, which is of high clinical importance if it is necessary to send the material to a remote diagnostic facility. It should be emphasised that molecular biology methods are necessary to determine the type of influenza virus as well as to perform strain sequencing when screening for candidates for optimal vaccine composition in a given season[12,14,15].

Conventional PCR

The method allows for detecting and replicating genetic material of pathogens using the polymerase chain reaction. PCR was first used in 1983 by Kary Mullis, an American biochemist, who won the Nobel Prize in 1993. Although DNA is always used as a PCR template, RNA may be used as starting material. The majority of respiratory viruses have their genetic information stored in the form of ribonucleic acid (RNA). RNA viruses are identified based on reverse transcriptase PCR (RT-PCR), which involves one- or two-step reverse transcription of RNA into complementary DNA (cDNA) using reverse transcriptase. Two-step RT-PCR involves transcription of RNA into cDNA in one reaction tube, followed by sequence amplification in another reaction tube. The two-step RT-PCR seems to show higher specificity and efficiency compared with its one-step equivalent. However, one-step PCR is more rapid, reduces the risk of sample contamination and increases the reproducibility of results[7,11,12]. Conventional PCR is increasingly replaced by RT-PCR. The RT-PCR detection of type A and B influenza virus in the Department of Influenza Research, National Influenza Centre at the National Institute of Public Health – National Institute of Hygiene is accredited by the Polish Center for Accreditation[9].

Real-time PCR

PCR consists of four steps. In the first step, primers are attached to complementary template sequences. This is followed by an exponential growth of the product during the exponential phase. In the logarithmic phase, reagents are gradually consumed and the reaction stops in the last phase known as the plateau phase.

Real-time PCR is a modification of conventional PCR. The method involves the use of appropriate dyes, probes and equipment to monitor product growth in each subsequent reaction cycle. The threshold cycle (Ct) or crossing point (Cp), which indicates the number of template replicates so that the fluorescence threshold (Ft) is achieved, is of key importance[16]. The higher the levels of template DNA, the faster the amplification curve will exceed the threshold value and reach a lower value. However, real-time PCR has its limitations, which may affect the accuracy of the measurement, ranging from improper isolation of clinical material and the quality of reaction substrates, the presence of reaction inhibitors to the type of analyser or human errors[17]. Undoubtedly, real-time PCR is considered to be a model example of quantitative PCR.

The use of qPCR in virology allows for the prediction of the infection status, identification of the stage of viral infection and monitoring of the efficacy of antiviral therapy used[1,7,12,13].

Real-time PCR is increasingly used in laboratory diagnostic tests due to faster and repeatable results as well as high sensitivity, even for small-volume samples. Real-time PCR significantly reduces the time of obtaining results (from several hours to several dozen of minutes) and establishing the diagnosis, which is of great importance in patients with influenza[5,9,14].

Real-time PCR is used for the detection of both type A and B influenza viruses as well as type A influenza viruses subtypes and type B influenza virus line in the Department of Influenza Research, National Influenza Centre, National Institute of Public Health – National Institute of Hygiene. Molecular biology methods are characterised by high sensitivity and specificity[5,9,12,18–20]. Influenza virus may be also detected post-mortem, based on autopsy specimens (spleen, heart, lungs)[21]. It should be emphasised that molecular biology methods allowed for the identification of the structure of A/H1N1/ influenza virus, which was responsible for the 1918–1919 pandemic[21].

In Poland, in addition to the Department of Influenza Research at the National Influenza Centre, National Institute of Public Health – National Institute of Hygiene (www.pzh.gov.pl, National Influenza Centre – nic@pzh.gov.pl), virology tests to confirm influenza infection are also performed in 16 provincial sanitary-epidemiological stations as well as some hospital and private laboratories.

At present, various methods of molecular biology used to confirm influenza infection limited the use of IF and ELISA due to their high sensitivity, specificity and waiting time for results confirming influenza infection, and thus the possibility of appropriate treatment initiation[5,7,11].

Immunofluorescence tests

Diagnostic tests use both direct immunofluorescence (DIF) and indirect immunofluorescence assays (IFA). DIF uses a specific antibody directly conjugated with a fluorochrome (fluorescein) and directed against specific antigens of a given virus, or unlabelled antibodies, followed by fluorochrome-labelled antiglobulin antibodies (indirect method)[5,7,11,12].

DOI: 10.15557/PiMR.2018.0032

IFA shows higher sensitivity than DIF. Commercial test, such as IF tests for respiratory syndrome, which allow for a full screening for seven different viruses: influenza virus type A and B, parainfluenza virus type 1, 2 and 3, adenoviruses, RSV, have also been developed, and the result is obtained in less than a dozen hours.

However, immunofluorescence requires rapid delivery of excellent quality unfrozen material to the diagnostic laboratory as well as storage and transport at an appropriate temperature[5]. The test should be performed as soon as possible to prevent cell damage. The analysis and interpretation of IF findings largely depend on the experience and competencies of laboratory personnel. Such tests are performed only in some sanitary-epidemiological stations.

ELISA tests

ELISA tests are immunoenzymatic tests to detect viral antigens using enzyme-conjugated monoclonal antibodies. The antigen is recognised directly by labelled antibodies or indirectly after an addition of specific unlabelled antibodies, followed by labelled antiglobulin antibodies. There are many commercially available ELISA tests, which are easy to use and interpret[5,11]. ELISA is performed only in some private laboratories.

Rapid diagnostic tests

Rapid diagnostics tests (the so-called bedside tests such as AB FLU OIA, Directigen Flu A, Directigen Flu A+B, QuickVue Influenza Test, Zstat Flu), which allow for direct visual detection of influenza virus A and B antigens within about 15–30 minutes, have been used as screening tools for a few years now. These tests differ in terms of, among other things, sensitivity, specificity and duration. Rapid diagnostic tests should be regarded as preliminary screening tests, which may be performed either by a doctor or a nurse during patient's appointment[5,10,22]. However, positive results should be confirmed using other virology tests, most often based on molecular biology, such as real-time PCR[10,22]. In Poland rapid diagnostic tests for influenza infection are performed in the Department of Influenza Research, National Influenza Centre, National Institute of Public Health – National Institute of Hygiene, some of hospital laboratories as well as private laboratories and private medical practices.

Influenza virus isolation

Despite the use of molecular biology methods for routine diagnosis (laboratory identification of influenza infection), virus isolation in 11-day-old chick embryos (multiplied in the allantois and/or amnion) and tissue culture is still the gold standard in the diagnosis of influenza virus[5]. In Poland influenza virus isolation in 11-day-old chick embryos and MDCK tissue culture (Madin–Darby Canine Kidney) is performed only in the Department of Influenza Research, National Influenza Centre, National Institute of Public Health – National Institute of Hygiene[5]. Currently, some virological laboratories in sanitary-epidemiological stations perform influenza virus isolation in MDCK tissue culture. Viral isolation in chick embryos is time-consuming and lasts up to 15 days of even longer, depending on the viral type[5,9,12].

Isolating and identifying influenza virus causing influenza in a given period, determining antigen similarity of viruses circulating in a given season as well as thorough testing using molecular biology methods, including viral sequencing, are essential for vaccine preparation, i.e. selecting candidates for vaccine antigen composition, and also for epidemiological reasons[12,14,15]. It should be noted that although 76 years have passed since the first authorisation for influenza vaccine, it is still produced in chick embryos[15].

It should be emphasised that isolation of influenza virus from a patient not only provides valuable information on antigen variant, i.e. the type or subtype of the virus circulating in the country and causing an increase in morbidity, but it is also important for the Global Influenza Surveillance and Response System (GISRS), in which Poland has been participating since 1957[9,12]. It should be also added that the World Health Organization established the Global Influenza Programme (GIP) during the Fourth International Congress for Microbiology held in Copenhagen in 1947. In its present form, six WHO Collaborating Centres for Reference and Research on Influenza located in London, Atlanta, Memphis, Tokyo, Beijing and Melbourne participate in the Programme.

At present, 144 national influenza centres in 114 countries participate in GISRS; however, there is an annual increase in the number of established national influenza centres[14]. In Poland, a total of 16 sanitary-epidemiological stations collaborate with the GISRS, with the Department of Influenza Research, National Influenza Centre functioning as the coordinator[9].

The role of GISRS is not only to determine antigenic changes in viruses circulating during the epidemic season, but also to warn about new subtypes of influenza virus type A, which was probably responsible for the influenza pandemic in the 21st century. Information from the National Centre for Influenza Surveillance in Hong Kong in May 9, 1997, when the H5N1 avian influenza virus for the first time in history broke through the species barrier and became pathogenic for humans (it is still circulating), was unquestionable evidence of the fundamental role of GISRS[23]. After extensive research using various methods of molecular biology, the isolated virus was named as highly pathogenic avian influenza (HPAI) A (H5N1) virus. Based on the international nomenclature system for isolated influenza viruses, the virus was coded as A/Hong Kong/156/97(H5N1) HPAI, where A stands for viral type, Hong Kong is the geographical place of isolation, 156 – the number of isolation, 97 – year of isolation (1997),

 DOI: 10.15557/PiMR.2018.0032

H5 – antigenic H5 hemagglutinin (H) subtype, N1 – antigenic neuraminidase (N) subtype.
In each country, influenza viruses isolated in a given epidemiological season are, following antigenic analysis, sent along with full documentation compliant with the requirements of World Health Organization to one of the six reference centres for further research. One of such centres is located in Poland in the Department of Influenza Research. The Polish National Influenza Centre sends influenza viruses isolated in the country to WHO Collaborating Centre for Reference and Research on Influenza, Francis Crick Institute (London, England) in each epidemiological season.

Collection of samples

The presence of influenza virus antigens in clinical groups may be confirmed by means of sample collection using: nasal and pharyngeal swab, nasopharyngeal lavage, middle ear effusion, aspirate from the nasal pharyngeal space, bronchial lavage, and cerebrospinal fluid. In some cases, the presence of the influenza virus may be also detected post-mortem, based on autopsy specimens (spleen, heart, lungs)[9,21].
Regardless of the choice of the method to confirm influenza infection, the correct result may depend on: appropriate smear collection, the time of sample delivery to a specialist facility, transport conditions (temperature), duration of disease symptoms at the time of the test, information about the medications used, e.g. anti-influenza agents.
Depending on the type of test, there are detailed descriptions of procedures for a sample taken from a patient – they are available on the laboratory's website, as is the case with tests performed in the Department of Influenza Research, National Influenza Centre, National Institute of Public Health – National Institute of Hygiene. To make things easier, we present the sequence of actions to be taken in order to familiarise with the procedure – from sampling to the price list: enter: 1) www.pzh.gov.pl; 2) Structure; 3) Division of the Deputy Director for Epidemiological and Environmental Safety; 4) Department of Influenza Research, National Influenza Centre; files, paragraph 6 – Instructions regarding the collection, storage and transport of clinical materials for diagnostic tests at the Laboratory of the Department of Influenza Research; paragraph 1 – Type of clinical material depending on test direction and methodology along with illustrations, how to collect material and a photograph of proper material collection; files, paragraph 7 – Order form for diagnostic tests in the laboratory of the Department of Influenza Research, National Influenza Centre, National Institute of Public Health – National Institute of Hygiene; Type of activity – Service, price list – Part I. Medical care services – virological, molecular and serological diagnostic tests[9,10].
In the case of rapid tests, the manufacturer's requirements should be followed. Material in the form of swab or lavage from the upper respiratory tract should be collected no later than 48 hours from the onset of clinical symptoms indicative of infection with influenza virus[10].

SEROLOGICAL DIAGNOSIS

In addition to virological tests, it is also possible to perform a serological evaluation allowing for serological confirmation of influenza infection based on the detection of increased patient's serum antibody levels[5,7,24–26]. For this reason, the procedure requires parallel tests in two samples of the patient's serum collected in the acute and convalescent period of the disease or possibly during the convalescence period, and again after a few weeks, when a decrease in the level of antibodies is observed.
Currently, the hemagglutination-inhibition (HI) assay is a routine test used by Polish clinicians to determine the level of anti-hemagglutinin antibodies following influenza infection or vaccination against influenza[25–29]. This test is based on the ability of anti-hemagglutinin antibodies to inhibit virus-induced agglutination of erythrocytes. The neuraminidase-inhibition (NI) assay, on the other hand, is used mainly in scientific research to assess the immune efficacy of vaccines, especially in high-risk groups, along with the hemagglutination-inhibition assay[25–29].
In Poland, both the hemagglutination-inhibition assay and the neuraminidase-inhibition assay are performed only in the Department of Influenza Research, National Influenza Centre, National Institute of Public Health – National Institute of Hygiene[10,25–30].
Serological tests to measure the immune response to the influenza virus are performed for a variety of reasons. They are used for diagnostic purposes, for assessing immunity after natural infection or after vaccination against influenza.
Tests measuring cellular immunity are not widely used in diagnostics, but they provide additional information on the body's defences[7,12].
It should be added that other serological tests have also been used, such as: complement fixation test, rapid enzymatic diagnosis (ELISA) or single radial haemolysis (SRH) – used in some research centres to measure antibodies against influenza virus. Currently, these tests are used only in scientific research[7,11,15]. Although they are useful in epidemiological studies, they can only confirm the diagnosis in a patient who has already had a severe phase of the disease. However, the diagnosis should be confirmed earlier, especially in seriously ill patients. The necessity to study the pairs of sera in the above-mentioned tests renders the diagnosis retrospective, and thus limits its usefulness in numerous clinical situations.

THE IMPORTANCE OF DIAGNOSTICS

Diagnosis of viral respiratory infection with particular emphasis on influenza has many advantages as it allows:
- to avoid antibiotic therapy in the absence of indications (antibiotics have no effects on influenza virus, but are used in bacterial superinfections);

DOI: 10.15557/PiMR.2018.0032

- to initiate appropriate treatment using currently available new generation anti-influenza agents, i.e. neuraminidase inhibitors (e.g. zanamivir, oseltamivir or peramivir) – caution is necessary to prevent the development of strains resistant to these inhibitors;
- to take some measures to prevent the infection from spreading (e.g. putting the patient in isolation, compliance with basic hygiene principles, etc.);
- to make hospital stay shorter;
- to reduce the cost of treatment in the case of influenza-induced complications;
- to debunk myths associated with vaccinations, which lead to their avoidance.

An early, correct and complete virological diagnosis of respiratory infection, with particular reference to influenza, is currently of great importance in terms of both medical and economic aspects[6,8]. It is also important for the detection of a new, unidentified pathogen, which has been confirmed many times, for example in 1997, 1999, 2003 and 2004[8,14].

At the turn of the 20th and 21st century, human infection with avian influenza virus, which broke through the species barrier, took place. These events drew attention to the serious threat of a new pandemic as well as to the need for enhancing the international influenza surveillance using appropriate diagnostic tools[8,12,14].

The high variability of the influenza virus should raise our awareness of the role and importance of diagnostic tests and convince the opponents about the absolute necessity of their use.

Conflict of interest

Authors do not report any financial or personal connections with other persons or organisations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

Funding/Support and role of the sponsor

Co-author Brydak L.B. – the study was partially conducted as a part of the 4/EM statutory project, NIZP-PZH.

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DOI: 10.15557/PiMR.2018.0032 PEDIATR MED RODZ Vol. 14 No. 3, p. 286–292