ACTA ABP BIOCHIMICA POLONICA

Vol. 61, No 3/2014
465–470
on-line at: www.actabp.pl

Review

Detection of the influenza virus yesterday and now

Agnieszka Woźniak-Kosek[1]✉, Bogumiła Kempińska-Mirosławska[2] and Grażyna Hoser[1]

[1]Centre of Postgraduate Medical Education, Laboratory of Flow Cytometry, Warsaw, Poland; [2]Department of the History of Medicine, Pharmacy and Military Medicine of the Medical University of Łódź, Łódź, Poland

Demographic changes and the development of transportation contribute to the rapid spread of influenza. Before an idea of a "person to person" spread appeared, divergent theories were developed to explain influenza epidemics in the past. Intensified virological and serological tests became possible after isolation of the human influenza virus in 1933. The first influenza virus detection methods were based on its isolation in egg embryos or cell lines and on demonstration of the presence of the viral antigens. Molecular biology techniques associated with amplification of RNA improved the quality of tests as well as sensitivity of influenza virus detection in clinical samples. It became possible to detect mixed infections caused by influenza types A and B and to identify the strain of the virus. Development of reliable diagnostic methods enabled fast diagnosis of influenza which is important for choosing an appropriate medical treatment.

Key-words: influenza, virus, detection method, diagnostics, PCR, viral respiratory tract infection.

Received: 11 February, 2014; revised: 19 May, 2014; accepted: 07 August, 2014; available on-line: 01 September, 2014

INTRODUCTION

The influenza virus is a cause of morbidity and mortality in patients with low immunity, but also in healthy persons. Influenza, as an acute infectious disease, was probably known in antiquity, as may be confirmed by the writings of Hippocrates and Livy from 412 B.C. (Harris, 1919; Hopkirk, 1913). Between 1173 and 1427 A.D. several possible influenza outbreaks were documented in different European countries, however, the first documented influenza pandemic was that of 1580. In the 19th century the greatest influenza epidemic was the one of 1889. At that time the etiological factor of influenza was not known. As influenza symptoms are not distinctive enough to be able to establish a diagnosis based solely on the clinical picture (Boivin *et al.*, 2000; Monto *et al.,* 2000), it is important to use reliable diagnostic tools, which is essential for hospitalized patients and those particularly at risk of complications from influenza. Correct diagnosis not only allows the avoidance of antibiotic treatment (unnecessary in the case of viral infection) but, most importantly, it guarantees effective treatment with antiviral drugs, such as the influenza neuraminidase inhibitors oseltamivir and zanamivir. These drugs are effective if applied preferably up to 36 hours from the onset of the symptoms (Nicholson *et al.,* 2000). Late drug administration may entail the risk of selection of resistant, difficult to eradicate strains. In such case the risk of post-influenza complications increases significantly. (Jong *et al.,* 2005; Moscona, 2005). Current diagnostic methods can confirm the presence of the virus genetic material in just 1.5 to 2 hours.

HISTORICAL ASPECT OF INFLUENZA DETECTION

Influenza epidemics were most likely known in antiquity. In the works of Hippocrates, who described different types of the so-called catarrh fevers under the name *febris catarrhalis epidemica*, we find it, as a disease with epidemic occurrence, characterized by unusual weakness, headache and high fever (Grant, 1782). In Europe, flu epidemics also probably occurred several times in the Middle Ages, for example, between 1173 and 1427 outbreaks were reported in Italy, Germany, France and the Netherlands (Rosen, 1993). The first documented influenza pandemic was that of 1580, which covered Europe, Africa and America, and was characterized not only by very high incidence, but also extremely high mortality (Flu Pandemics web side). In the 18th century there were two pandemics (1729–1733, 1781–1782), and in the 19th century there were three (1830–1833, 1847–1848, 1889–1891) — the largest being in 1889, described as "flowing like a wave around the world in just a few months" (Hopkirk, 1913; Shope, 1931). In the 20th century the greatest pandemic was recorded in 1918–1919. At that time, the etiological agent was still not known. Subsequent pandemics, far less severe but still serious, took place in 1957, 1968, and 1977.

After the 1889 and 1918 pandemics it became evident that influenza is an infectious and contagious disease. An important role in epidemiological studies was played by the London physician H. F. Parsons, who analyzed the spread of the illness and suggested that influenza spreads by means of "person to person" (Parsons, 1893). The pandemic of 1918-1919 caused an unprecedented intensification of research on the etiology of influenza. This ultimately led to the isolation of the human influenza virus in 1933.

Some light on the nature of the flu was shed by epidemiological observations, such as those that were made during the epidemic of 1889, which connected morbidity with age or gender. The way of spreading the flu was unclear for doctors at that time. Although at the end of the 19th century the theory that microbes spread influenza from person to person increasingly held sway, some

✉e-mail: kaj12@poczta.fm

people still explained its nature within the framework of the so- called miasmatic theory.

Three periods can be distinguished in the history of research on influenza The first, up to1889, when the diagnosis of influenza was based solely on clinical symptoms; the second, between 1889 and 1933, when in addition to the clinical symptoms researchers began to take into account the results of laboratory tests, such as serological tests; and the third, beginning in 1933, when the influenza virus diagnostics were introduced and developed. (Kilbourne, 1987).

Laboratory studies, initiated by the pandemic of 1889, focused on the determination of pathological changes in different organs caused by influenza, and detection of the etiological agent. The research material consisted of samples (pieces of tissue) taken from people who had died from the flu and blood or secretions, such as sputum, collected from patients (Nowiny Lekarskie, 1890). Isolation of the etiological agent causing influenza in humans was made in 1933 by using ferrets intranasally infected by nasopharyngeal lavage fluid collected from patients with influenza (Smith *et al*., 1933). The following years were a time of intense development of influenza virology, namely determining its types, subtypes, strains, construction of virus particles, and research on vaccines against influenza. Concerning the pandemic of 1889, some studies indicated that it could have been caused by influenza A virus subtype H2N2 (Masurel *et al*., 1973; Oxford, 2000); others, that it was caused instead by the virus H3N8 (Taubenberger *et al*., 2007). There is no doubt, however, that the pandemic of 1918 was caused by the H1N1 virus, which circulated for several subsequent years. In February 1957 Asian flu began in China, spreading from there to Singapore, then to Hong Kong. The H2N2 virus was isolated in May in Japan, in June in the U.S., and in July in the U.K. The first wave of the epidemic, however, occurred as late as in October 1957, the second wave in January 1958. Both waves were associated with increased mortality (Cox *et al*., 2000). Interestingly, serological tests performed during an outbreak in 1957 indicated the presence of antibodies recognizing the new H2N2 virus isolated in 1957, in people aged 70–90 (Shope, 1958). In 1968 a new virus subtype, A/H3N2, emerged, and the Hong Kong pandemic occurred. In 1977 this virus was replaced with the virus subtype A/H1N1. The division into subtypes and strains was possible by the intensive development of research in the field of molecular biology.

LIMITATIONS AND OBJECTIVES OF CLINICAL DIAGNOSTICS FOR THE DETECTION OF INFLUENZA VIRUSES

The clinical symptoms of influenza are often nonspecific, and thus the disease can be diagnosed with certainty only by laboratory tests. However it should be kept in mind that the evaluation of the clinical material may be limited by improperly collected and handled samples. Material for the tests may be swabs collected from the nose, nasopharynx or throat, a bronchial lavage, ear exudate, or biopsy material (Kim *et al.,* 2013). About 200 types of various respiratory viruses are circulating in the population. Therefore, a close attention should be paid to the type and manner the material is being collected, its proper storage and transport to a specialized laboratory.

Modern diagnosis of respiratory tract infections

The most common infectious agents of the respiratory tract are viruses (Cox *et al*., 2000; Grubek-Jaworska, 2012; Kim *et al.,* 2013;). An important task for the clinical physician is to recognize such cases and to distinguish them from the bacterial infections. This is not an easy task because the symptoms associated with respiratory infections are not specific for bacterial or viral etiology. In such cases the availability of modern, rapid, sensitive, and specific diagnostic tests for pathogen identification helps to apply appropriate therapeutic strategies and reduces unnecessary use of antibiotics.

Conventional diagnosis

Viruses that cause respiratory infections, in particular the influenza virus, are propagated in embryonated chicken eggs. Currently this method is rarely used in Poland because of the difficulty to obtain embryos of adequate quality. When cell culture lines are used, the most popular are: (i) MDCK adherent cell lines, cultured from epithelial tissue taken from dog kidney, (ii) the VERO cell line, derived from epithelial tissue taken from African green monkey kidney, (iii) the Hep-2 line, from human laryngeal carcinoma cells, (iv) the A549 cell culture line, derived from a line of human lung cancer cells, and (v) the MRC5 line, derived from the lung tissue of a 14-week human fetus (Fong *et al*., 2000; Huang *et al*., 2000, Kim *et al.,* 2013). The standard culture methods used in the diagnosis of influenza offer almost 100% sensitivity, but the waiting time for results is rather long. However, according to the recommendations, the targeted therapy should be initiated up to 36 hours after the onset of the disease symptoms. As a result, the classic methods for the isolation and propagation of the influenza virus in cultures are used in the selection processes of vaccine strains in each epidemiological season (Brydak, 2008) and rather seldom for diagnostic purposes. In Poland, the National Influenza Centre, Department of Influenza Research, National Institute of Public Heath, National Institute of Hygiene uses the method of isolation of virus in embryonated chicken eggs and in cell cultures.

Serological diagnosis

These methods are based on the estimation of antibodies in the patient's serum. This requires the parallel tests performed on serum samples collected from a patient at the acute and convalescent phase of the disease. Serological tests include complement fixation and haemagglutination inhibition reaction (Kim *et al.,* 2013). The first method is used for retrospective diagnosis of influenza virus infection. The flu virus has 2 antigens that bind the complement. Antibodies for the antigen S (nucleocapsid — specific type for influenza virus) appear during the first week of the disease and persist for 3–6 months. Antigen V represents the surface antigens specific for influenza strain (haemagglutinin and neuraminidase). Antibodies to this component appear about a month after infection, and can be detected up to 2 years later. This method is suitable for testing large-scale Abs persistence, but is not used in routine diagnostics. For serological diagnosis, most laboratories use haemagglutination inhibition reaction. This assay is based on the ability of antibodies (anti-HA) to inhibit the influenza virus-

Table 1. Characteristics of Rapid Influenza Diagnostic Tests (Information for Clinical Laboratory Directors http://www.cdc.gov/flu/professionals/diagnosis/rapidlab.htm)

Procedure (Manufacturer/Distributor)	Influenza Virus Types Detected	Approved Specimens	Test Time
3M™ Rapid DetectionFlu A+B Test(3M) Moderately complex test — requires specific laboratory certification	A and B	nasopharyngeal/ swab/aspirate nasal wash/aspirate	15 minutes
Alere Influenza A & B(Alere)	A and B	Nasal swab	15 minutes
BD Veritor System for Rapid Detection of Flu A+B (Becton Dickinson) Requires use of a separate analysis device	A and B	nasopharyngeal/ swab/nasal swab	10 minutes
BD Veritor System for Rapid Detection of Flu A+B Moderately Complex (Becton-Dickinson) Requires use of a separate analysis device Moderately complex test — requires specific laboratory certification	A and B	nasopharyngeal wash/aspirate/ swab	10 minutes
BinaxNOW® Influenza A&B(Alere)	A and B	nasopharyngeal/ swab/ nasal wash/aspirate	15 minutes
BioSign® Flu A+B (Princeton BioMedtech) Moderately complex test — requires specific laboratory certification	A and B	nasopharyngeal swab/aspirate/ wash, nasal swab	15 minutes
Directigen™ EZ Flu A+B (Becton-Dickinson) Moderately complex test — requires specific laboratory certification	A and B	nasopharyngeal wash/aspirate/ swab/throat swab	15 minutes
OSOM® Influenza A&B (Sekisui Diagnostics) Moderately complex test — requires specific laboratory certification	A and B	nasal swab	10 minutes
QuickVue® Influenza Test (Quidel)	A or B	nasal wash/aspirate/swab	10 minutes
QuickVue® Influenza A+B Test(Quidel)	A and B	nasopharyngeal swab/nasal wash/aspirate/swab	10 minutes
SAS™ FluAlert A&B (SA Scientific) Moderately complex test — requires specific laboratory certification	A and B	nasal wash/aspirate	15 minutes
SAS™ FluAlert A (SA Scientific)	A only	nasal wash/aspirate	15 minutes
SAS™ FluAlert B (SA Scientific)	B only	nasal wash/aspirate	15 minutes
Sofia Influenza A+B (Quidel) Requires use of a separate analysis device Immunofluorescence assay	A and B	nasopharyngeal aspirate/swab/ wash nasal wash	15 minutes
TRU FLU® (Meridian Bioscience) Moderately complex test — requires specific laboratory certification	A and B	nasopharyngeal aspirate/swab/ nasal wash	15 minutes
XPECT™ Flu A&B (Remel/Thermofisher) Moderately complex test — requires specific laboratory certification	A and B	nasal wash/swab throat swab	15 minutes

induced agglutination of erythrocytes. The highest serum dilution which still inhibits the agglutination of blood cells is called the titre of anti-HA antibody for a given strain of influenza virus.

Rapid diagnostic tests (RIDT-rapid influenza detection test) constitute the second group of tests used to determine the immune response of the body upon contact with influenza viruses. These assays are based

Table 2. Primers
Primer sets used for one-step RT-PCR for human influenza surveillance (London WHOCC; May 2011) http://www.who.int/influenza/resources/documents/diagnostic_recommendations/en/

Type/subtype	Gene fragment	Primer	Sequence	product size
Influenza type A	Matrix (M)	M30F2/08 M264R3/08	ATGAGYCTTYTAACCGAGGTCGAAACG TGGACAAANCGTCTACGCTGCAG	244bp
Influenza A(H1N1)2009 virus	HA	HKU-SWF HKU-SWR	GAGCTCAGTGTCATCATTTGAA TGCTGAGCTTTGGGTATGAA	173bp
Influenza A(H3N2)virus	HA	H3A1F3 HARUc	TGCATCACTCCAAATGGAAGCATT ATATCGTCTCGTATTAGTAGAAACAAGGGTGTTTT	863bp
Influenza A(H5N1) virus	HA	H5-1087F H5-1231R	CAGGGAATGGTAGATGGTTGGTA CGGCCTCAAACTGAGTRTTCAT	153bp
Influenza B Victoria	HA	Bvf224	ACATACCCTCGGCAAGAGTTTC	
lineage		Bvr507	TGCTGTTTTGTTGTTGTCGTTTT	284bp
Influenza B Yamagata lineage	HA	BYf226 BYr613	ACACCTTCTGCGAAAGCTTCA CATAGAGGTTCTTCATTTGGGTTT	388bp

on enzymatic immunoassays or immunochromatic reactions, and may differ, among others, in terms of sensitivity and specificity, positive and negative predictive value, the time of performance and reading test results, the type of biological material recommended for the test, as well as the scope of analysis. Some tests only detect influenza A virus, others — both influenza type A and type B, as shown in Table 1.

Molecular diagnosis

The genome of influenza virus consists of eight separate segments of single stranded RNA with negative polarity. This structure creates the possibility of reassortment, thereby promoting the continuous evolution of the virus (Webster *et al.* 1992). The most significant changes occur in the genes encoding the two major glycoprotein surface antigens: HA (segment 4) and NA (section 6). The high rate of mutation and the huge variation within these genes led to the development of a dozen or so subtypes of HA and NA. Individual subtypes include different variants, and this further complicates the development of targeted PCR, specific for one particular subtype of influenza virus. Using the most conservative sequences in the genome for primers' designation, RT-PCR can be used to detect all strains of influenza virus (Kim *et al.,* 2013). However, using primers specific for certain types of HA and NA allows determination of the subtype of the influenza virus. Table 2 and 3 show some examples of primers for detection of influenza virus type A and B using RT-PCR and Real-Time PCR.

RNA amplification enables early detection of influenza virus genetic material, even on the day of the onset of symptoms, before the level of antigens permits detection allowed by other methods. Most respiratory viruses have RNA genomes, and therefore, in the case of the diagnosis of these viruses, the amplification of the characteristic fragments of the genome must be preceded by reverse transcription of he genetic information into the DNA sequence (producing a complementary DNA — cDNA). This modification of PCR by adding a reverse transcription step is called RT-PCR (Reverse Transcription-PCR). RT-PCR may be performed in two variants: a one-step variant (one-step RT-PCR), when the reverse transcription and PCR are performed in a single tube, or two-step (two-step RT-PCR), where the conversion of RNA into cDNA and the amplification of the tested sequences are separate steps, the two-step reaction being more sensitive than the one-step reaction. On the other hand, the one-step RT-PCR reaction is faster, and minimizing the number of steps reduces the risk of contamination and increases the reproducibility of the obtained results (Kim *et al.,* 2013).

Another method used is Real-time PCR: polymerase chain reaction with monitoring of the amplified product in real time. It is a technique that has revolutionized clinical microbiological diagnostics. This method combines in one reaction tube the PCR technique with fluorescent labeling of the amplified product. The analysis takes considerably less time than classical PCR, as it takes about 1–2 hours. This method is much simpler to perform and is associated with less effort. The diagnostic efficiency of this method in the case of viral infections is comparable to serological methods or cultures (Espy *et al.*, 2006, Grubek-Jaworska, 2012). The advantage of Real-time-PCR in comparison to the classical PCR is also due to the possibility of quantitative pathogen evaluation. This method is currently preferred due to the possibility of monitoring the amount of the reaction product in each cycle of the reaction, significant automation of the technique and lower time consumption, as well as less human effort. The Real-time PCR method allows the estimation of the level of viremia; it can also be used to monitor the progress of treatment in patients.

In the case of microorganisms which can colonize the respiratory tract, the problem of the usefulness of molecular methods in the detection of etiology of infections is not yet resolved. An important novelty is

Table 3 Primers and probes
Primer sets used for one-step Real Time-PCR for human influenza surveillance (London WHOCC; May 2011)
http://www.who.int/influenza/resources/documents/diagnostic_recommendations/en/

Type/subtype	Gene fragment	Primer	Sequence
Influenza type A	Matrix (M)	MP-39-67F MP-183-153R MP-183-153R	CCMAGGTCGAAACGTAYGTTCTCTCTATC TGACAGRATYGGTCTTGTCTTTAGCCAYTCCA 5′-(FAM)-ATYTCGGCTTTGAGGGGGCCTG-(MGB)-3′
Influenza A(H1N1)2009 virus	HA	NIID-swH1 TMPrimer-F1 NIID-swH1 TMPrimer-R1 NIID-swH1 Probe2	AGAAAAGAATGTAACAGTAACACACTCTGT TGTTTCCACAATGTARGACCAT 5′-(FAM)-CAGCCAGCAATRTTRCATTTACC-(MGB)-3′
Influenza A(H3N2)virus	HA	NIID-H3 TMPrimer-F1 NIID-H3 TMPrimer-R1 NIID-H3 Probe1	CTATTGGACAATAGTAAAACCGGGRGA GTCATTGGGRATGCTTCCATTTGG 5′-(FAM)-AAGTAACCCCKAGGAGCAATTAG-(MGB)-3′
Influenza A(H5N1) virus	HA	H5HA-205-227v2-F H5HA-326-302v2-Rev H5-Probe-239- RVa3	CGATCTAGAYGGGGTGAARCCTC CCTTCTCCACTATGTANGACCATTC 5′-(FAM)-AGCCAYCCAGCTACRCTACA-(MGB)- 3′
Influenza type B	NS	NIID-TypeB TMPrimer-F1 NIID-TypeB TMPrimer-R1 NIID-TypeB Probe1	GGAGCAACCAATGCCAC GTKTAGGCGGTCTTGACCAG 5′-(FAM)-ATAAACTTTGAAGCAGGAAT-(MGB)-3′

the introduction of the GeneXpert system (Cepheid, USA) for the diagnosis of infections, including respiratory tract infections, which can identify, among others, the influenza viruses. The Xpert FLU panel allows for the detection of the influenza virus directly in the sample, distinguishing the virus types A and B and subtype A/H1N1/.

Presently, the multiplex PCR seems to have a highest diagnostic potential. Multiplex PCR is a modification of classical PCR, which uses many primers allowing the amplification of more than one fragment of DNA or RNA in a single reaction. This method allows for the identification of multiple pathogens in a single test. Limitation of the practical application of this technique in upper respiratory tract infections is that there might be some difficulty in differentiating between pathology and colonization, or in determining the pathogen dominant in the disease in case of multi-pathogen infections. (Greiner *et al*., 2001; Grubek-Jaworska, 2012; Templeton *et al*., 2004). Tests based on the multiplex PCR method enable simultaneous detection in nasopharyngeal secretions of influenza virus types A and B, parainfluenza type 1–3, hMPV, RSV, rhinovirus, enterovirus, adenovirus, coronaviruses (229E, OC43), and the bacteria — *Mycoplasma pneumoniae* and *Chlamydia pneumoniae*.

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