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Virologic diagnosis of influenza: detection of infection and assessment of immunity[1)]

Abstract: A variety of methods are available for virological diagnosis and immunity assessment of influenza. For virus detection, the clinical specimen is usually the respiratory swab. In terms of sensitivity, nasopharyngeal aspirates are superior and sputum specimens are inferior. The diagnostic method of choice is RT-PCR of viral DNA sequences coding for matrix and nucleoprotein, which define influenza virus types A, B, C, or coding for hemagglutinin and neuraminidase spikes on the viral envelope identifying influenza virus A subtypes and strains. Gene-specific primer probes are commercially available. PCR takes 1–2 h after the arrival of the clinical specimen. Cell culture based virus isolation combined with intracellular antigen detection needs 1–2 days, but is regarded as gold standard. Transport of the swabs in an ice chest is recommendable. Direct antigen detection in the clinical specimen by the use of enzyme-linked immunosorbent assay (ELISA) or immunofluorescence test (IFT) is the most rapid (bedside) method, but of inferior sensitivity. Therapy resistance analysis is done by genotyping subsequent to PCR amplification or by the cell culture method (phenotyping). Antibodies due to influenza virus are produced during the second week after infection and may confirm or disprove virological diagnosis. ELISA and IFT apply nucleoprotein or matrix proteins as antigens and differentiate between the Ig classes IgA (IgM) and IgG indicating an acute or passed infection. Complement fixing antibodies do not persist. Influenza virus immunity is assessed by neutralization assay or – more simply – by hemagglutination inhibition in a type-, subtype- and even variantspecific manner.

Keywords: antibody test; immune assessment; influenza; resistance analysis; virus detection.

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Introduction

Among the respiratory virus infections, influenza is a more severe disease sometimes requiring intensive care of the patient. Concerning elder people, in every influenza epidemic an excess mortality is recorded by public health authorities. Due to this relevance, soon after the discovery of the infectious agent in the thirties of the last century, a lot of laboratory methods have been developed to detect the influenza virus in clinical specimens and to check the antiviral immune status. In this review, the current methods of influenza diagnostics is compiled and discussed in terms of a "state of the art" [1–4].

Influenza virology

Influenza viruses belong to the big family of orthomyxoviridae (old Greek "ortho"=regular, "myxos"=mucus). The viral structure and replication have been extensively investigated (diameter about 100 nm), as outlined in Table 1 and in Figures 1 and 2 [4, 5]. Influenza viruses are classified in several *genera*; three of them are endemic in humans as influenza virus *types* A, B, and C. While B and C are human-specific, type A is prevalent in water birds (ducks, geese, gulls) with many *subtypes*. Some variants are also endemic in humans, pigs and other mammalians. The single-stranded RNA genome of these viruses consists of eight segments. Sequence analyses of the whole genome have revealed many variances, since the viral RNA polymerase does not copy very exactly. The mistake rate of RNA replication amounts about 1:1000 nucleotides. Those point mutations affect all parts of the virus structure. Envelope mutations make the virus escape the host immunity selecting special variants (*antigen drift*). The main envelope structure is a glycoprotein spike, which makes the virus adsorbing to the membrane of the respiratory target cells. Sialic acid (SA=N-acetyl-neuraminic acid) was identified as receptor on the membrane. After adsorption, the virus is uptaken by the cell via endocytosis. By virus-cell adsorption, erythrocytes can be agglutinated in vitro. So, the viral anti-receptor was called hemagglutinin (H).

Table 1 Influenza virology.

3 influenza virus types: A, B, (C)
Influenza viruses B and C: Only pathogenetic for humans, no subtypes
Influenza A: Zooanthroponosis (birds), several subtype variants are adapted to a mammalian host.
The RNA genome of the influenza viruses undergoes many point mutations resulting in variants. This *drift may* lead to "immune escape", change of pathogenicity and of adaptation to hosts.
Since the RNA genome is segmented, simultaneous infections with different subtypes or variants may induce the formation of "reassortants" (*shift*) and enhance the *drift*.
There are a lot of virus infections with influenza-like symptoms (parainfluenza, RSV, metapneumo-, corona-, rhino-, entero-, adenoviruses)

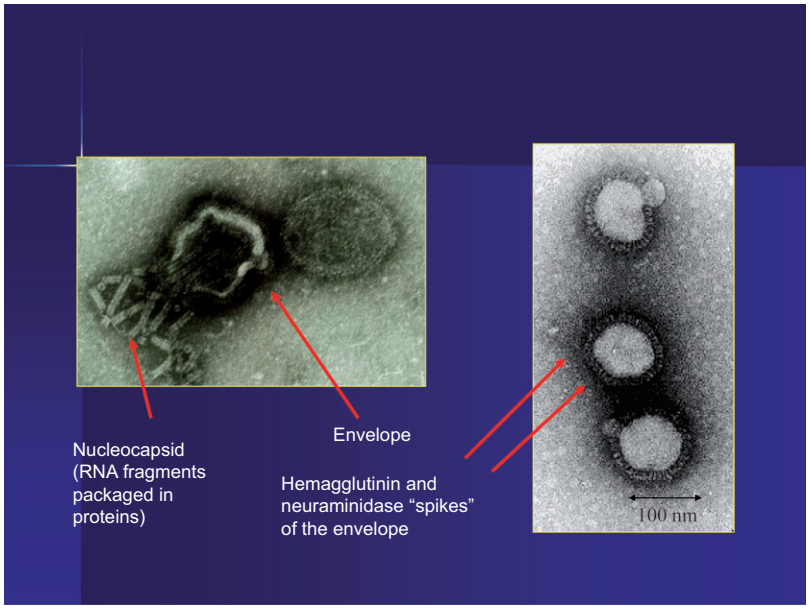

Figure 1 Elektron micrograph of influenza virus.

A second kind of envelope spikes serves as neuraminidase (N) destroying the cell receptor. This enzyme activity prevents readsorption and releases the progeny virus from the cell membrane after replication or from cell fragments in the mucus. Virus mutations do not only drift, but also *shift*: If two different virus variants coinfect and replicate in the same cell, RNA segments from both parent viruses may be reassorted and packaged in a new genome. Coinfection and RNA segment *reassortment* of different virus subtypes can (rarely) produce a new subtype. Because of its big host reservoir, influenza A viruses (IAV) reveal a much bigger variability than B and C. Up to date, IAV variants are classified in 16 H- and in 9 N-dependent subtypes based on cross neutralization by the use of specific antisera. So, 16×9=144 serologic subtypes may theoretically exist. Up to date, 105 serosubtypes have been discovered in birds. Three of them reveal human-specific variants: H1N1, H2N2, and H3N2 (numbering had started with research on human influenza). Current subtyping is much more exactly done by RNA sequencing of H and N genes. Molecular biologic and serologic classifications may not completely coincide, as was seen in the swine-origin influenza 2009. The virus was identified as IAV subtype H1N1 by RNA sequencing. However, because of mutations in the sialic acid-binding site of this H1, the cross neutralization by conventional anti-H1 was very small. Thus, the new H1N1, which had been previously endemic in pigs and jumped over the species barrier, was termed H1N1/2009, swine-origin H1N1 or H1N1v (v=variant). The vaccine is derived from the first isolate H1N1v (California) [5–8].

Pathology and immune protection

The influenza pathology depends on several factors of virulence, as efficiency of virus adsorption to the target cells, interaction of virus replication and cell metabolism,

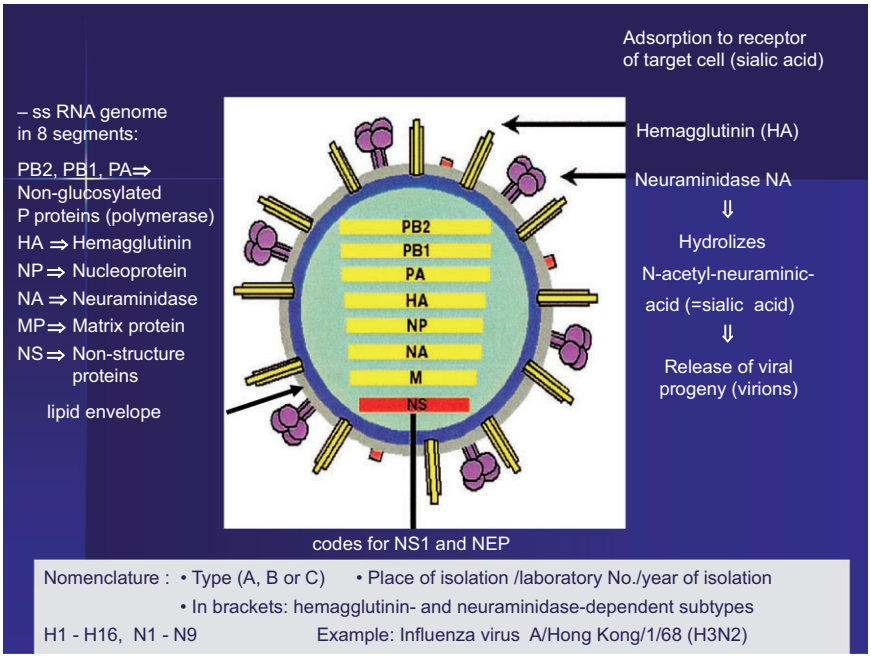

Figure 2 Structure of influenza A virus [out 4].

release of viral progeny from the infected cells [4, 5], and of pathogenic (inflammatory, immunologic) host reactions to the infection [9]. The first crucial step of infection is the interaction of H on the viral envelope with the SA receptor on the cell membrane. In the different animal species, there are two SA modifications depending on which C atom of the SA galactose molecule is bound to the membrane polysaccharide. On the cells of the upper respiratory tract in humans and other mammalians, a C1-6 connection is established, in water birds a C1-3 connection. IAV variants of water birds reveal H modifications which are hardly adapted to the C1-6 SA of mammalians. Such IAV strains are not able to infect mammalians easily except the pig, since the respiratory cells of this animal express both SA modifications. Thus, pigs are susceptible for avian and human IAV strains and may be the site of reassortment of interspecies virus genes thus serving as dangerous virus vessel between birds and humans, when a genetic shift is followed by an H gene adapting drift [5, 6].

The worldwide spread of avian Flu caused by IAV H5N1 since 1997 has shown, that humans are only seldom infected by avian IAV except under the settings of industrial livestock farming when big amounts of virus may be produced by an infected chicken flock and reach the lower respiratory tract of a predisposed man, whose cells express the SA C1-3 modification, to [5]. Such infection frequently results in a lethal pneumonia [10, 11]. Since influenza viruses bypass immunity by genetic drift and shift, there is no complete herd protection against current influenza viruses in the population. An influenza virus infection can only be prevented by specific antibodies directed against the viral envelope structure, which interacts with the cell receptor like key (SA) and lock (apical site of H). Partial immunity is sterically mediated also by antibodies to neighborhood H sites. However, every H structure is affected by genetic drift, as already mentioned. The hemagglutination inhibition test (HI) is the method of choice to evaluate the individual status of anti-influenza immunity. Its results reveal a very high accordance with the classic cell culture-supported infection-neutralization test (NT) [12]. Beside the anti-H antibodies, also anti-N antibodies can stop or reduce the infectivity of influenza viruses (in [13]). Synthetic neuraminidase blockers, which act against the catalytic site of the enzyme, are successfully used as therapeutical antivirals [2]. Specific antibodies are produced not until or after the second week of influenza disease. Healing is mediated by the formation of cytolytic T lymphocytes (CD8), which attack and destroy the infected cells. The presentation of matrix and RNA nucleoproteins on the membrane of infected cells via the MHC I system is highly immunogenic for this immune response. The elimination of infected cells diminishes the virus production independent of the H/N subtype, because internal structural virus components are not controlled by the pressure of immune selection [14]. So, they determine the virus type. In this way, the cell-mediated immune response can influence the competition between IAV subtypes (beside mechanisms of the innative immune system as natural

Table 2 Mortality of influenza A pandemics.

Year		Subtype	Mortality
1918	Spanish Flu	H1N1	40 m.
1957	Asiatic Flu	H2N2	4 m.
1968	Hong Kong Flu	H3N2	2 m.
1978	Russian Flu	H1N1	< 0.5 m.
2009	Mexico Flu	H1N1v	ca. 15000
2010	Cocirculation of (H1N1), H1N1v and H3N2		

m, million people.

killer cells, interferons etc.), e.g., spread of H1N1 before and after H2N2 1957–1978, s. Table 2).

Detection of influenza virus in clinical specimens

Classic influenza is characterized by typical symptoms (Table 3). In the individual case, diagnosis can be made only by virologic investigation. Several methods are available [15]. Gold standard is the virus isolation by the use of special permanent cell cultures (Table 4). A transformed cell line prepared out of canine kidney tissue is mostly used (Madin-Darby canine kidney, MDCK). 48 h post inoculation with material out of the clinical sample the infected cells have produced enough viral antigen to be detected by immunofluorescence or immunoperoxidase test. Cytopathic effects (CPE) subsequent to infection are often missed in the microscopic examination. However, CPE can be indirectly shown by the adsorption of chicken erythrocytes to the infected cells and confirmed by hemadsortion inhibition via specific antibodies. Clinical specimen of choice is the deep nasal swab or the nasopharyngeal aspirate. Pharyngeal swabs and sputum yield less virus. Virus detection in bronchial lavage provides a pathognomonic test result. However, this material sampling is too expensive in labor for routine diagnostics, of course. To conserve and transport the clinical sample, special fluids have been developed and introduced. Otherwise, also physiologic NaCl or cell culture fluids can be applied. If the clinical specimen cannot be immediately transported to the laboratory, the material should be ice-cooled. A long-term store needs at least –20°C. The infectivity of the patient comes to its end, when the disease heals, in the most cases 1–2 weeks post infection, in children and immunocompromized people often later [14]. Cell culture-supported virus propagation can be applied to test antivirals (phenotypic resistance test). Because it is rather time-consuming, this test plays no role in the routine patient management.

Detection of influenzaviral RNA genome by the use of RT-PCR in the clinical specimens above mentioned is hardly not less sensitive than virus isolation procedure [15, 16]. In the modern modification of "real-life" PCR, the investigation is ready about one hour after the specimen has arrived in the laboratory (Table 5). This RNA detection is less dependent on an optimal specimen collection and transport as virus isolation. However, if more time than two hours is needed for the transport, patient's material should be cooled or frozen as described above. For the efficacy of PCR, it is crucial to choose the right gene probes as reaction primers. A lot of information is easily available by the internet (Table 6). Influenza virus types, subtypes, and strains can be reliably identified by RNA amplification of sequences in the genes of matrix and nucleoproteins, of H and N spikes [16, 17]. Similar to HIV,

Table 3 Characteristics of influenza: Symptoms and clinical lab results.

- Respiratory disease caused by influenza virus type A or B
- Incubation period: 1–3 (–7) days
- Symptomatology: high fever, athralgia, nasopharyngitis, tracheitis; sometimes: Bronchitis; seldom: Primary pneumonia.
- Disease period: 1–2 weeks, recovery: many weeks
- Influenza is frequently the "pacemaker" of bacterial infections in the respiratory tract (or vice versa) sometimes causing pneumonia or spreading to other orgns (heart).
- Clinical laboratory: CRP+, ESR+, leucopenia/relative lymphocytosis
- Bacterial superinfection: CRP++, ESR++, granulocytosis

Table 4 Performance profile of influenza virus isolation procedure.

- Virus isolation in cell culture via intracellular antigen detection
- Time needed: 48 h
- Specificity: nearly 100%
- Sensitivity: dependent on specimen collection and transport up to 80%
- Specimens: Nasal/nasopharyngeal swab, sputum, (water for throat rinsing). Special collection swabs are commercially available.

Table 5 Performance profile of influenza virus RNA detection by the use of RT-PCR.

- RT-PCR with special gene primer probes (MP, H, N gene sequences) for the detection of virus type, subtype, variant
- Time needed: 1–3 h
- Specificity: 100%
- Sensitivity: >95%

Table 6 Gene primer probes for the detection of influenza virus RNA by the use of RT-PCR [out 16, 17].

Type	Gene locus	Sequence
A	M	5′ CAT ggA ATg gCT AAA gAC AAg ACC
A	M	5′ AAg TgC ACC AgC AgA ATA ACT gAg
B	HA	5′ AgA CCA gAg ggA AAC TAT gCC C
B	HA	5′ CTg TCg TgC ATT ATA ggA AAg CAC
H1SW	HA	5′ ACA Agt TCA Tgg CCC AAT CAT gAC TCg

HI1SW, swine-origin IAV HIN1v 2009.

HBV, and HCV, automate-supported sequencing of cDNA in those genes delivers information on the emergency of therapy resistant virus strains (genotypic resistance test) and provides an insight into infection chains in the population [18, 19].

Even more rapid than PCR is the direct detection of influoenza viral antigens (M, NP, NS1 in Figure 2) in clinical specimens by the use of immunoassasys as immunofluorescence or ELISA. The specimen may be smeared and fixed to a slide. Alternatively, antibody-coated microvials or beads are used to catch the viral antigen out of the transport fluid of the swabs (Table 7). Several test systems are commercially available. The usage is so simple that they may serve as "bed-side" tests. While their specificity is satisfying, their sensitivity is limited (in [15, 20]). This may be less relevant, if influenza emerges as epidemic. However, in the individual case the infection cannot be reliably excluded.

Antibody tests

For the detection of influenza virus antibodies, a lot of tests have been developed:

The biologic methods, as infection neutralization (NT), hemagglutination inhibition (HI), complement-fixation (CFT), characterize the antibody function, but are time- and labor-consuming. Modern immunoassays, e.g., ELISA or indirect immunofluorescence test (IFT) produce a physicochemical test signal. ELISA results can be easily quantified and standardized. Furthermore, Ig class differentiation of antibodies is provided (IgG, IgA, IgM). For the diagnosis of acute influenza, all antibody tests play no big role, since seroconversion is seen usually only 10–14 days after infection. Nevertheless, they may confirm or disconfirm a passed influenza, which has not been virologically investigated [15, 21].

Table 7 Performance profile of influenza virus antigen detection.

- Antigen test with marker-conjugated antibody probe (IFT, ELISA) for the direct investigation of specimens without amplification procedure
- Time needed: 15 min (rapid test, bed-side test)
- Specificity: >95%
- Sensivitivity: 50–80% (kits are commercially available)

The first and classic antibody test was established by virus neutralization in cell cultures (formerly in infected laboratory animals or embryonated chicken eggs).

The test needs at least two days, even if combined with antigen detection in the infected cells. So, the method serves only to assess the immunity. Much more rapid is the usage of erythrocyte suspensions (chicken, guinea pig, human blood group 0) instead of cell culture. The erythrocytes are not infected, but agglutinated by virus adsorption. Before testing on hemagglutination inhibiting antibodies, each serum specimen must be absorbed with vibrio cholerae suspension providing neuraminidase as *receptor destroying enzyme* in order to eliminate free SA receptors from cell fragments. This material might compete with the erythrocyte SA receptors for anti-H antibodies and mock a positive HI result. Since HI had been developed 1942, this method is the standard test for assessment of immunity against influenza virus [12]. Its test result depends on which influenza virus H type, subtype or strain is applied. Antibody cross reactions between virus strains of the same subtype must be ruled out by titrations. Determination of antibodies against N, which confer also some immunity, is scientificly performed only [15].

For the routine diagnostics, antibody detection and quantification is done by modern immunoasays or CFT using type-, but not subtype-specific viral matrix- or nucleoprotein as antigens (Mp, NP). Since these proteins are more immunogenic than the H (or N) glycoproteins, seroconversion may be detected a little bit more rapid (but not earlier than one week after the onset of disease). Before the advent of commercially available immunoassays, CFT was a widely used standard method of influenza virus serology. Complement-fixing antibodies do not persist with higher titer values. So, a titer value 1:>20 may be of diagnostic relevance [22]. However, a relatively acute or recently passed influenza virus infection can be proved only by a significant titer rise (Table 8). The solid-phase immunoassays (ELISA, IFT) provide antibody test results differentiating Ig classes. IgA antibodies indicate a relatively recent infection or vaccination. IgA tests are more used than IgM tests for the diagnosis of respiratory virus infections. IgG antibodies indicate a passed influenza virus infection in a type-, but not subtype-specific

Table 8 Performance profile of serum antibody tests for influenza diagnostics and assessment of immunity.

Serum antibodies are detectable as early as one week after onset of influenza disease. Diagnosis is confirmed by seroconversion and/or significant titer rise. Tests:
– CFT: Type-specific, lower sensitivity, but detection of recently produced antibody titers (1:>20), which decline in the next year.
– HI and NT: more sensitive and specific than CFT, influenza virus type and subtype-specific; assessment of immunity (protection HI titer 1:>20).
– ELISA is simply and automate-supported performable. Like CFT only influenza virus type-specific. Differentiation of Ig class: IgG test indicates seroprevalence of an influenza virus type. IgA (IgM) indicates a relatively recent infection.
– IFT is more work-consuming than ELISA. By the use of stabilized erythrocytes coated with influenza full virus beside type- also an approximative subtype-specificity is reached.

way. The negative IgG test excludes the infection prevalence [21, 23, 24]. A special IFT has been developed, which does not work with infected and fixed cells, but with full virus coated to stabilized erythrocytes. With some restriction, this test determines subtype-specific IAV antibodies, too. Drift variants cannot be identified in this way [15, 21].

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