Chapter 1

Influenza Diagnosis with a Specific Emphasis on the M2e Antigen as a Diagnostic Tool

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/64786

Abstract

The therapy, observation, inclusiveness, and preclusion of related diseases all influence the diagnosis of influenza. Particularly, the pandemic duration and diagnosis time for influenza are extremely important. After the appearance of symptoms, antiviral medication must be initiated within 48 h. Cell culture, real-time polymerase chain reaction (PCR), flow cytometry, direct and indirect immunofluorescence methods, and the quick diagnosis test are all valuable approaches for the diagnosis of influenza. Different instruments, different time durations for the results, and different specialists characterize all these approaches. Antigen selection is of critical importance with regard to the specificity and sensitivity of these methods, especially the serological and rapid diagnosis tests. M2e, the highly conserved external domain of the influenza A M2 protein, is a potential differential diagnostic marker for influenza virus infection. This chapter reviews the studies that use M2e as a diagnosis agent, and it illuminates the role and importance of M2e in the diagnosis of influenza.

Keywords: M2e, diagnosis, peptide, antigen, virus culture, rapid immunochromotographic test, serological test, ELISA, PCR

1. Introduction

Influenza viruses can cause respiratory infections, and it is a major cause of morbidity and mortality worldwide [1]. Influenza is typically a mild disease that lasts for 1–2 weeks in most people [2], although in some cases it can be fatal [3]. The likelihood of developing complications is higher in certain risk groups, such as people with chronic health problems, children under 2 years old, and the elderly [4]. While 45–77% of those hospitalized due to influenza are under 65

years old, the patients who are older than 65 years are the most likely (60–90%) to die as a result of influenza [5–8].

The variable nature of the influenza has already caused several pandemics. The accurate and rapid diagnosis of influenza is therefore of great importance to the effective management of epidemic and pandemic periods [4]. As there are several respiratory pathogens that may cause clinical symptoms similar to those caused by influenza, a final diagnosis is difficult for doctors to establish [9]. As a result, sensitive and rapid diagnosis methods are needed to confirm the clinical diagnosis of influenza, as well as to improve the quality of monitoring systems. A variety of laboratory methods for the diagnosis of influenza are available (**Table 1**). Each of these methods has both advantages and disadvantages, and some or all of these factors may affect the selection of an appropriate diagnosis method [2].

Diagnosis method	Average acquisition time	Mechanism	Application opportunity	Sample required
Viral culture	3–7 days	Virus isolation	Limited	Bronchoalveolar lavage Nose and throat swab Nasopharyngeal aspirate
Molecular tests	1–2 days	RNA detection	Limited	Post-mortem tissue Bronchoalveolar lavage Nose and throat swab Nasopharyngeal aspirate
Rapid immunochromatographic tests	15–30 min	Antigen detection	Widespread	Bronchoalveolar lavage Nose and throat swab Nasopharyngeal aspirate
Serological tests	2 days	Antigen/antibody detection	Widespread	Serum

Table 1. Comparison of the diagnostic methods available for the influenza A virus.

In this chapter, the methods that have developed and used in the diagnosis of influenza will be evaluated in terms of their principles, sensitivity, specificity, time, advantages and disadvantages, and the antigens used for the diagnosis of influenza A.

2. Diagnosis methods for influenza A

There are four basic methods available for the diagnosis of influenza [10], each of which is detailed in **Table 1**.

http://dx.doi.org/10.5772/64786

2.1. Virus cultures

2.1.1. Classic virus cultures

Symptoms such as the deformation of cell morphology, cell necrosis, pouring from the cell location to the cell culture, and fusion in cells that are infected with the virus are, depending on the virus proliferation, referred to as the cytopathic effects (CPE) [11].

Virus cultures that use primary rhesus monkey kidney cells (PMK) or Madin-Darby canine kidney cells (MDCK) are commonly accepted to be one of the "gold standards" for laboratory diagnosis. Whether or not the cells are infected with the virus is determined by the cytopathic effects in the cell cultures and hemadsorption using immunofluorescence monoclonal antibodies against influenza [10].

2.1.2. Rapid shell vial virus cultures

Coverglasses are used in the rapid shell vial cell culture method to passage the cell lines onto them. The specimens are inoculated after the monolayer cell lines occur. The coverglasses are then stained with cause-specific FITC-marked monoclonal antibodies for about 24–48 h. Cytopathic effects are not expected with this method, unlike in standard tube cultures [12].

Influenza isolation with the rapid shell vial culture method provides an advantage with regard to its simplicity and speed when compared to traditional culture method [13]. Influenza shell vial cultures show results within 1–3 days after virus inoculation [12] (**Figure 1**).

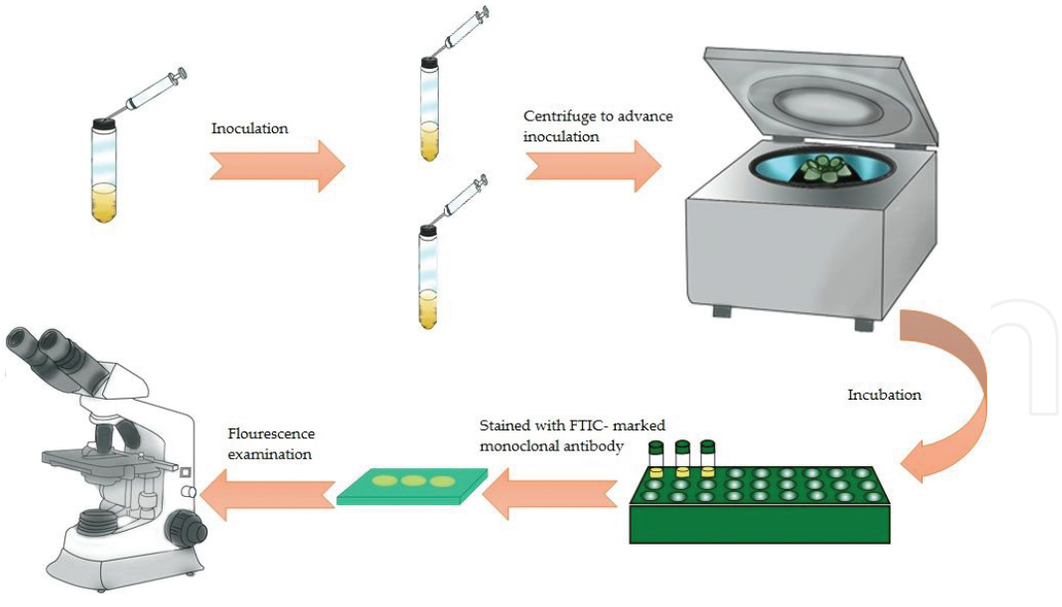

Figure 1. Schematic presentation of shell vial method.

The specificity of the rapid shell vial test is 100%. Although the time necessary to obtain the results is much shorter in this method than in traditional cell cultures, in many cases, the time is actually insufficient to begin optimal antiviral treatment [10].

2.2. Molecular methods

The genome of the influenza viruses consists of eight different parts of single-stranded negative sense RNA. This structure changes continuously and it contributes to the evaluation of the virus [14]. Major changes occur in the genes encoding the two major surface glycoproteins, namely the hemagglutinin (HA) and neuraminidase (NA) antigens. The HA antigens play a role in the connection of the virus to the cell, NA plays an essential role in release and spread of progeny virions, following the intracellular viral replication cycle [15]. There are 18 different hemagglutinin subtypes and 11 different neuraminidase subtypes, H1–H18 and N1–N11, respectively [16]. The high mutation rate causes the development of subtypes [17].

The extreme genetic variability of the influenza viruses leads to challenges in the design of molecular-based diagnosis tests. Most conserved sequences in the genome used to determine the RT-PCR primers can be used to identify individual strains of influenza virus [12]. A PCR that has been modified by the addition of the reverse transcription step is known as a reverse transcription-PCR (RT-PCR). In addition, the use of specific primers for significant types of HA and NA antigens can aid in the identification of subtypes of influenza viruses [17].

A RT-PCR can be performed in two different ways: (1) the one-step approach (reverse transcription and PCR), which is performed in a single tube; and (2) the two-step approach, which involves the transformation of RNA to cDNA and the amplification of the tested sequences. The two-step reaction process is more sensitive when compared with the one-step reaction. On the other hand, the one-step RT-PCR reaction is fast, and the minimal number of steps reduces the risk of contamination and so improves the reproducibility of the obtained results [12].

Many RT-PCR tests of the subtypes of influenza have shown greater sensitivity than other rapid diagnosis tests and conventional cell cultures. The RT-PCR activity does not change depending on the age of the patients. The rotation period of the RT-PCR for influenza is between 1 and 2 days. In addition, molecular tests require considerable skill and expertise, and they should hence be integrated into the laboratory processes [10].

2.3. Rapid immunochromatographic tests

Rapid diagnosis kits are immunochromatographic methods that include the use of monoclonal antibodies against preserved antigens or nucleoproteins that are localized with a membrane or impregnated onto a strip of influenza A or A and B [18].

Briefly put, according to these methods, the respiratory specimen is primarily treated with an extraction buffer and it is then applied to a filter paper or test strip depending on the test format. If influenza viral antigens are available, a visible color change is generated by the reaction of the antigens and influenza-specific monoclonal antibodies [19].

The sensitivity of the rapid diagnostic kits is approximately 70%, depending on the particular test kit used, the patient's age, and the sample collection time [20]. The specificity of the rapid

diagnostic kits ranges from 76 to 100% [21]. In addition, many kits can distinguish between the influenza A and B strains, although they cannot subtype further [20].

Influenza A has a higher sensitivity in the rapid diagnosis test kits when compared with influenza B. Such rapid diagnosis kits are very convenient, and they have a high positive predictive value because of the spread of influenza in the community [21]. The most important advantage of these tests is the fact that they can provide results in approximately 10–30 min [10].

However, during periods of low influenza activity, the predictive value of the rapid diagnostic kits is also low, and false positive results are more likely. Therefore, the use of these tests during periods of high influenza activity is suggested [21]. In addition, more specific testing methods must be performed on patients who present with negative results according to the rapid influenza diagnostic kits during high influenza periods. The major disadvantage of these tests is their limited shelf life of approximately 1–2 years. The inadequate collection of samples and the misinterpretation of test strips by inexperienced personnel may also lead to errors [10].

2.4. Serological tests

Serological methods are all based on determining the antibody response in the sera or specific antigens or gene sequences of influenza virus. Specific, sensitive, and validated serological assays can be used for diagnosis of influenza, the identification of the source of infection, the epidemiology studies, and the identification of asymptomatic cases. Serological methods have also been utilized at prescreening of influenza disease [22].

The immunology of tested population and sensibility and specificity of the tests have major role on interpret results of serological tests [23]. Each type of serological methods have own advantages, disadvantages, and unique characteristics [24]. The serological diagnosis of influenza infection is based on agar gel immunodiffusion, radioimmunoassay, immunofluorescence antibody tests, hemagglutination inhibition, enzyme-linked immunoassay (EIA), complement fixation, and an increase of specific antibody titer between acute and healing serum samples as measured by neutralization tests [10, 17, 25].

2.4.1. Direct immunofluorescence antibody test (DFA)

In this method, antibodies are labeled with fluorescent compounds called fluorophores. Fluorophores are generally organic molecules with cyclic structure. Fluorescein and rhodamine are examples of the most used molecules.

In this method, the antigen in the suspected material is fixed on slides and specifically labeled antibodies placed onto the antigen. Fluorescence labeled (FITC) "monoclonal antibodies" are considered positive due to the luminescence in the presence of agent in patients' samples [12, 25–27]. DFA test is developed for antigen detection in tissue or body fluids and used successfully for diagnosis of influenza. Among children who have high fever and spread lots of influenza viruses, DFA sensitivity is commonly higher [26] (**Figure 2**).

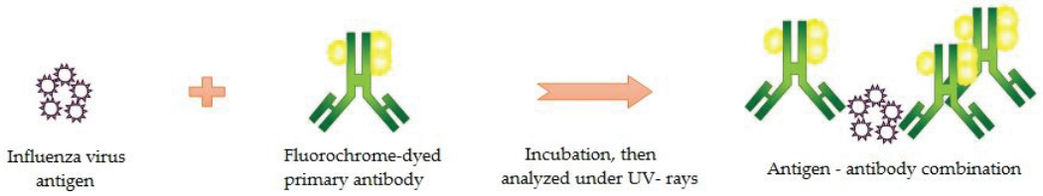

Figure 2. Schematic presentation of direct immunofluorescence antibody test.

2.4.2. Indirect immunofluorescence antibody test (IFAT)

In an indirect immunofluorescence assay, antibodies against influenza labeled with fluorescence dyes are used [10, 27, 28]. Influenza A virus particles are fixed on a slide and then the suspected serum is added. FITC conjugated anti-human IgG antibodies are added and examination is made under a microscope [29]. If there is a homologous antibody against the virus on the slide in the influenza suspected serum, a yellow-green glow is seen under a microscope (i.e., a positive reaction) [10, 12].

In principle, the serum antibodies react with immunofluorescence allow for the rapid diagnosis. The sensitivity of the IFA test is 70–90%, while the specificity is above 90% for diagnosis of influenza [20]. There are individual differences in the reporting methods for the different immunofluorescence tests, since this test faces the issue of subjectivity in the reading of slides. To overcome this problem, the IFAT should be reported by experienced clinical laboratorians [30, 31] (**Figure 3**).

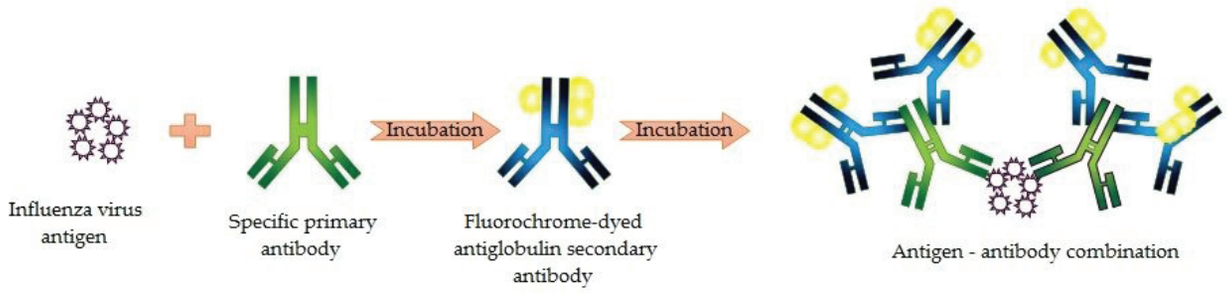

Figure 3. Schematic presentation of indirect immunofluorescence antibody test.

2.4.3. Radioimmunoassay (RIA)

Radioimmunoassay test is used to search for both an antibody against influenza and virus or viral antigen, immunoglobulins are conjugated with radioactive material (radioisotopes ^{14}C, ^{125}I, etc.). In positive reactions, the immune complex that occurs when the influenza antigen and specific antibody couple gains radioactivity. This radioactivity is determined by special counters (such as a gamma counter detector). When the results are evaluated according to a curve known as the standard curve, an overall result can be seen. The degree of determined

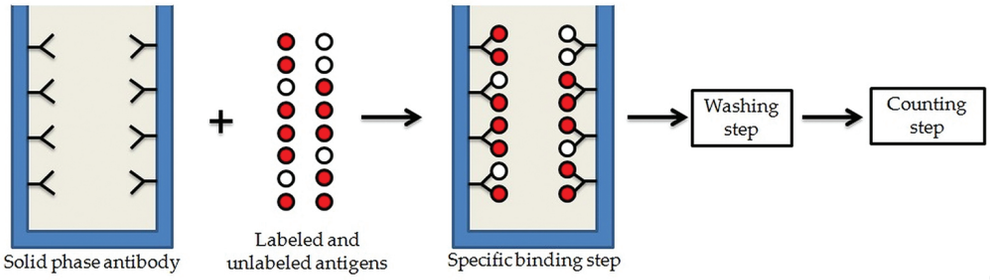

Figure 4. Principle of RIA. Labeled antigen competes with patient antigen for a limited number of binding sites on solid-phase antibody.

radioactivity could be measured in this way. In negative reactions, radioactivity cannot be detected [27, 32].

RIA is a very sensitive and precise technique for detection low amounts of analytes. Otherwise, the health hazard of radioactive substances is most important limitation of all RIA techniques due to disposal problems, short half-life, and the need for expensive equipment [31] (**Figure 4**).

2.4.4. Agar gel immunodiffusion (AGID)

The AGID detects antibodies against all influenza A viruses, regardless of subtype. The principle of the AGID test is the simultaneous migration of antigen and antibodies toward each other through an agar gel matrix. Influenza antigens and antibodies form a precipitate in to gel matrix and precipitation line is visualized. Various parameters such as electrolyte concentration, pH, and temperature can affect precipitate formation [33]. Also, concentrations of the antigen or antibodies cause shift the location of the line [34]. The immunodiffusion method can be performed in three different ways, namely simple diffusion (the Oudin method), unidirectional diffusion (the Mancini method), and bidirectional diffusion (Ouchterlony) [35]. The advantages of this method are simplicity, low cost, and inessential specialized laboratory equipment [36].

2.4.5. Neutralization test

The neutralization assay determines that patients' antibody can neutralize the infectivity of a given influenza virus strain. Sera that contain specific neutralizing antibodies prevent the cytopathic effects of influenza virus [27]. Influenza viruses cause CPE via proliferation in cells form plaques (i.e., the CPE focus) on nutrient agarose-coated cells [19]. If the virus is cultured on monolayer cells after being stirred with antiviral homologous antibodies formed against itself and then left at room temperature for 30–40 min, plaques either do not form (i.e., plaque neutralization) or else the number of plaques decreases in those cells coated with nutrient agar (i.e., plaque reduction). A reduction in the number of plaques of 50% or more is considered a positive reaction. If the virus encounters a homologous antibody carrying serum, then plaque formation is seen because the neutralization does not occur [12].

The influenza subtypes can be defined by using a type-specific antiviral immune serum. The neutralization reaction is also utilized as a protection test in animals [27].

The neutralization test has several advantages which it can identify functional, strain-specific antibodies in sera [37]. However, it cannot be used routinely since it is time-consuming and laborious.

2.4.6. Complement fixation (CF) test

The complement fixation test (CFT) is one of the classical influenza diagnostic assays, which mainly detects IgG antibodies [38].

In this test, the primarily influenza suspected serum (titrated), then the known viral antigen (titrated), and later the complement (fresh guinea pig serum titer) are added. They are thoroughly mixed and then allowed to stand. At this stage, if a homologous antibody against influenza is present in the serum, the antibody couples with the antigen and the complement binds with this complex. If there is no antibody present, coupling with the antigen does not occur and hence the complement cannot connect and so remains free. A mixture known as the hemolytic system (hemolytic serum + sheep red blood cells) is added to the tubes to determine whether or not the complement is attached. If there is no hemolysis in the erythrocytes, the complement is held in the first stage or, in other words, in the antibody-antigen complex. This indicates the presence of an antibody in the influenza suspected patient's serum (i.e., a positive reaction) [12, 39].

CFT is useful test for diagnosis of acute virus infection, however, it is quite complex, less sensitive, very labor intensive, and is not suitable for automation.

2.4.7. Hemagglutination (HA) vs. hemagglutination inhibition (HI)

The hemagglutination inhibition test is an important diagnostic tool in certain infections especially influenza. The hemagglutinin protein on the surface of the virus may cause agglutination in the presence of erythrocytes [40]. Blocking of this aggregation by specific antibodies in the patient's serum is named as hemagglutination inhibition test [37].

The HA agglutination test is the conventional method for the determination of influenza viruses, while the HI test is commonly used for typing [40]. If the sera from individuals suspected of carrying the disease have virus-specific antibodies against the virus, then these antibodies prevent HA by neutralizing the HA property of the virus when the antibodies and the virus come into contact with each other. In test tubes, erythrocyte aggregate occurs on the bottom in the form of a round point [19].

In the HI test, the numbers of antibodies within the serum are proportional to the amount of HI titers. The HI titers in the serum may remain at the initial dilution if there is less antibody present, although the titers in the serum will reach different dilutions if there is too much antibody present [39, 41].

The hemagglutination inhibition assay is a reliable, relatively simple, and inexpensive technique to antigenically characterize isolates of influenza viruses [37]. Whereas the HI test

is very useful in epidemiological surveys, it is not suitable for routine diagnosis of influenza [42]. Nowadays, this assay is widely used and is replaced by more modern immunoassays [38].

2.4.8. Enzyme-linked immunosorbent assay (ELISA)

In enzyme immunoassays, enzyme-conjugated antibodies are generally used for detection of viral antigens. These groups of tests, antigen or antibody are bound to solid phase as microtiter plates, nitrocellulose membranes, and magnetic latex beads [31]. Patient serum is added to antigen bounded solid phase and an enzyme-labeled antiglobulin is added. If anti-influenza antibody is present in patient's serum, enzyme-labeled secondary antibody reacts and chromogenic substrate causes color change [27].

The specific antigens as HA, NA, NP, and M protein of influenza are widely used ELISA. The sensitivity of EIA varies between 64 and 78% [43]. Because of the high specificity, sensitivity, simplicity, and low cost, ELISA is one of the most common used immunoassays in the clinical laboratory [31].

3. Antigens that are used in diagnosis

The methods used in the diagnosis of influenza (except for viral cultures) are based on the determination of the antigen of the virus, the determination of antibodies against the antigen in the patient, or the determination of the gene region of the selected antigen as in molecular methods [40]. In all these methods, the target antigenic structures are used as important selection criteria that affect the specificity and sensitivity of the method. Nucleoproteins and neuraminidase are used in the viral commercial diagnostic kits (rapid tests) popular in commercial studies and academic research. Due to the virus undergoing changes, the use of these antigens in the diagnosis tests affects the obtained results. For this reason, it is also important to use the conserved regions of influenza A in diagnosis [44].

Therefore, in this part of the chapter, the M2 protein, the most conserved structure of influenza A, will be evaluated in terms of its diagnostic importance by considering its biological functions.

3.1. Definition of M2e protein

In influenza A viruses, one of the most important structural proteins is matrix protein 2 (M2) [45]. The M2 protein, the internal membrane protein, is made up of an ectodomain part, which is a single membrane spanning domain located at the N-terminus, and a cytoplasmic tail, which is found in the C-terminal [46].

While the N-terminus domain is 24 amino acids long, part of it is located at the outer side of the membrane surface (M2e), while the 54 amino acids long C-terminus is situated in the cytoplasmic side and the remaining 19 amino acids sequence the lipid bilayer [46] (**Figure 5**).

From the first human influenza A strain that was isolated in 1933 to the present day, no amino acid mutation has been found in the extracellular domain of the M2 protein [47]. In all subtypes

of the influenza A viruses, the extracellular domain of the M2 protein, M2e, is significantly conserved with an unchanged SLLTEVET (residues 2e9) sequence at the N-terminal [48]. The M2e protein consists of a homotetramer structure with disulfide linkages that are held together by noncovalent interactions [49]. The M2e protein is conserved in all types of influenza A viruses. Despite appearing in small amounts on the surface of mature virions, this protein is majorly expressed on the surface of infected cells [46].

The peptide epitope of the influenza A virus M2e protein is: Met-Ser-Leu-Leu-Thr-Glu-Val-Glu-Thr-Pro-Ile-Arg-Asn-Glu-Trp-Gly-Cys-Arg-Cys-Asn-Asp-Ser-Ser.

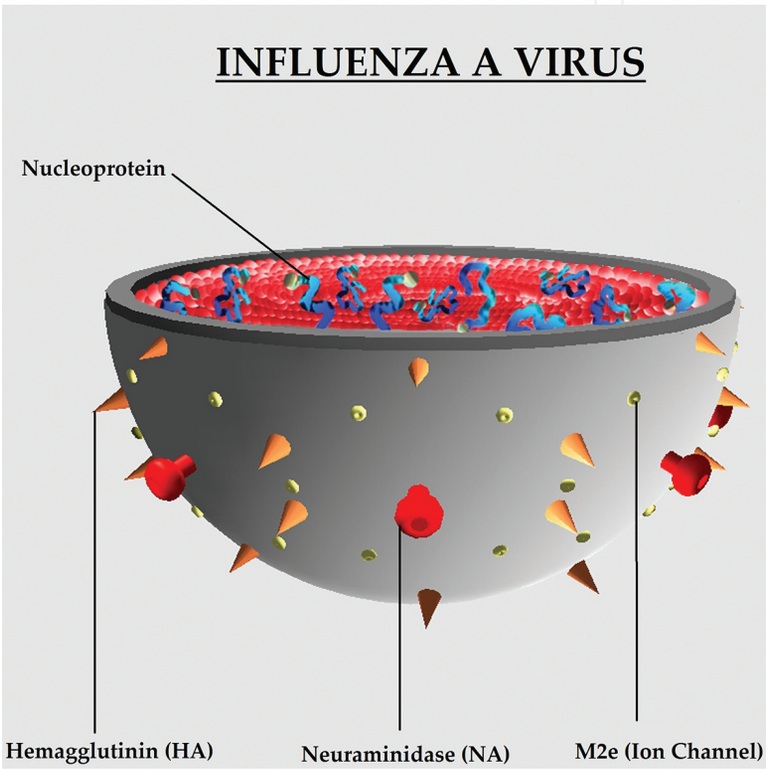

Figure 5. Schematic presentation of influenza A virus structure.

3.2. Biological function of M2e

The main functions of the M2e protein are: (i) functioning as a proton channel; (ii) playing a role in viral replication; and (ii) defending the maturation of HA and structural integrity [48].

The M2e protein is not required for viral replication, but during viral disintegration, it does act as an ion channel that allows the entry of protons into the virions [50]. M2 also plays an effective role in virus morphogenesis and assembly.

M2e stabilizes the newly synthesized HA molecules by adjusting the pH toward the secretion pathway [51]. M2 transport protons reduce the interior pH of the virions via a receptor-mediated endocytosis procedure during the entry of the virus [52].

3.3. Immunological responses against M2e

Mucosal and systemic immunity play a role in the body's resistance to infection. Influenza-specific lymphocytes have been detected in the blood and lower respiratory secretions of influenza patients [53]. A short-term primary cytotoxic T-cell response can be detected after 1–2 weeks (infection with influenza viruses leads to virus-specific B cell as well as T-cell responses) [54]. Antibodies occur against type-specific internal proteins, NA, and M1, as well as against viral surface glycoproteins such as HA and NA. The neutralizing antibodies against HA are the first immune constituents to protect the host from infections caused by the influenza viruses [55, 56].

M2 is a significantly expressed structural protein on the surface of infected cells. M2e is a small portion with a low immunogenicity in its native form [52]. The relatively small size of M2e and its low abundance in virions when compared to other glycoproteins like HA and NA are possible explanations for the low reactivity of M2e [57]. Anti-M2e antibodies can develop in some infected patients [52]. The host's adaptive immune system attracts the virus. At the same time, the infection of humans with influenza A viruses stimulates a weak anti-M2 antibody response for a short duration of time [58]. The anti-M2e antibodies' seroprevalence is increased with age, which is an auxiliary factor in this pre-existing immunity against M2 [59].

M2e-specific antibodies act through the antibody dependent cell cytotoxicity, and the innate or complementary immune system promotes the killing of infected cells. Specific M2e antibodies prevent the release of viral particles toward the extracellular fluid or else stimulate the uptake by phagocytic cells through the Fc receptors via connecting to the viral cell [44, 52].

3.4. Studies that have used M2e in the diagnosis of influenza

Ingrole et al. chose to use M2e in their study based on its preserved region. The use of an elastin-like particle led to the increase in size of the produced antibody. M2e can easily be recognized by the M2e-specific antibodies due to the conjugation of M2e and ELP (elastin-like particles) [60].

Khurana et al. conducted an experiment based on HA2 (488–516), PB1-F2 (2–75), and M2 (2–24) peptides, which have the most conserved regions of the H5N1 strain. In their study, experiments were carried out with ELISA and rapid diagnostic kits, and the effects of these peptides were investigated. In the study that used rapid diagnostic kits, the attachment to the HA2 and M2 peptide bands was observed to be positive in the serum of infected patients. In the ELISA study, there was no reactivity against H5N1 in patients who were vaccinated with a vaccine that did not include the M2e peptide [61].

Denis et al. investigated the use of the papaya mosaic virus as a carrier of the conserved and small amount M2 peptide instead of HA and NA peptides in a vaccine study. Papaya mosaic virus conjugated virus-like particles have been observed to increase the production of the antibody against the M2 peptide. Specific bindings were observed against M2e in both ELISA and MCDK cell culture experiments [62].

Wolf examined the effect of M2e-based multiple antigenic peptides on the production of antibodies via ELISA and ELISPOT techniques. The specificity to the M2e peptide was measured in antibody secreting cells by ELISPOT assay and, as a result, a higher formation of immunoglobulin levels was observed [63].

Hadifar et al. used an ELISA method based on the distracted intravenous access (DIVA) test against H5N1 in their study. The M2e antigenicity of the monomers that form when infected in a natural way to be limited is developed with the DIVA test method. The use of the tetramer structure of M2e instead of the M2e monomer increased the efficiency of the ELISA and other tests mentioned in this study [64].

Tarigan et al. used the single M2e peptide (sp-M2e) ELISA and multiple antigenic forms of the M2e ELISA methods, and the sensitivity of these tests in the diagnosis of M2e was compared. The degrees of M2e were measured by ELISA in order to use the M2e as a target in the DIVA test during pandemics. Although both ELISA techniques have specificity in the diagnosis of the M2e peptide, the MAP-M2e ELISA technique has higher specificity and sensitivity in the diagnosis of M2e [65].

Black et al. determined the influenza A (H2N2) M2 expression of recombinant baculovirus via an indirect immunofluorescent antibody assay (IFA) in a study conducted in 1993. The formation of an antibody against M2e was determined using the EIA test [54].

4. Future aspects

In a study conducted by the US Food and Drug Administration (FDA), the analytical performance of the 11 most widely used influenza diagnostic tests on the market was evaluated in a comprehensive manner. In experiments conducted using 23 different influenza subtypes that have been in circulation recently, although most brands could detect the viral antigen in samples that included a high concentration of influenza virus, it was found that detection based on subtypes of the virus is limited in lower concentrations. It was further suggested that physicians should be careful of negative results when using rapid diagnosis tests for the diagnosis of influenza [66].

Peptide-based enzyme immunoassays are widely used in the sero-diagnosis of bacterial and viral infections. These tests can be easily applied due to their enhanced advantages, namely simplicity, specificity, sensitivity, and relative inexpensiveness.

Despite the genetic differentiation of the influenza A virus, it has been shown that the peptide region formed by the 24 amino acids of the M2e protein located on the surface of the virus is the most conserved region in all strains, and it has therefore been used as a potential diagnosis marker in various studies [56, 57, 67, 68]. Although M2e is normally found in small quantities on virus particles, it is secreted abundantly on the cell surface infected by the virus and, in particular, it will provide a significant advantage in the detection of the disease during pandemic periods [61]. Therefore, diagnosis tests that are developed based on M2e will be able to detect different subtypes of the influenza viruses.

Acknowledgements

The authors would like to thank The Scientific and Technological Research Council of Turkey (TUBİTAK) for their support the named "Development of Rapid Diagnostic Kit with Immunochromatographic Method of Depending on IgY Antibody Specific in (M2e) Peptide for Diagnosis of Influenza A Infection" (Project No. 115S132).

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