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REVIEW ARTICLE

Evolving Gene Targets and Technology in Influenza Detection

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Abstract Influenza viruses cause recurring epidemic outbreaks every year associated with high morbidity and mortality. Despite extensive research and surveillance efforts to control influenza outbreaks, the primary mitigation treatment for influenza is the development of yearly vaccine mixes targeted for the most prevalent virus strains. Consequently, the focus of many detection technologies has evolved toward accurate identification of subtype and understanding the evolution and molecular determinants of novel and pathogenic forms of influenza. The recent availability of potential antiviral treatments are only effective if rapid and accurate diagnostic tests for influenza epidemic management are available; thus, early detection of influenza infection is still important for prevention, containment, patient management, and infection control. This review discusses the current and emerging technologies for detection and strain identification of influenza virus and their specific gene targets, as well as their implications in patient management.

1 Introduction

Influenza is a well-known, highly contagious disease that has recurring epidemic outbreaks every year causing significant morbidity and mortality, and appears to have afflicted human populations since ancient times. The exceptional severity of the first influenza pandemic of the twentieth century, the 1918–1919 "Spanish flu," which caused ~50 million deaths, accelerated the research effort in identifying the causative agent [1–3]. These research efforts have advanced knowledge of the epidemiology of influenza viruses in many different aspects.

Influenza viruses belong to the *Orthomyxoviridae* family, which currently contains three known types: A, B, and C. Both influenza A and B viruses cause the same spectrum of illness, such as fever, chills, severe malaise, myalgias, headache, sore throat, and dry cough, with influenza A viruses being the most prevalent cause of cases during the annual flu epidemic, while influenza C virus causes sporadic upper respiratory track illnesses in children and adolescents. Influenza A viruses also infect a wide variety of animals, including swine, horses, and birds (both domestic and wild). The natural reservoir of influenza A viruses is thought to be aquatic birds. Influenza B viruses circulate mostly in humans, while influenza C viruses infect humans, swine, and potentially dogs [1, 4–6].

Influenza viruses are enveloped viruses with a segmented negative strand RNA genome. Influenza A and B viruses contain eight distinct segments, while influenza C viruses contain seven segments. For influenza A and B viruses, the RNA segments 1–3 encode for transcriptase complex; segment 1 for polymerase protein PB2, segment 2 for polymerase protein PB1, and segment 3 for polymerase protein PA. For influenza A viruses, segment 2 also encodes PB1-F2 (an virulent factor), and PB1-N40 (unknown function) [7, 8]. The two major antigens of the virus are encoded by segments 4 (*hemagglutinin*, HA) and 6 (*neuraminidase*, NA). Segment 5 encodes for nucleocapsid protein (NP), segment 7 codes for two matrix proteins, M1 and M2 (BM2 for influenza B), and segment 8 encodes two proteins NS1 and NS2. Influenza B virus segment 6 encodes an additional protein, NB. Influenza C virus has similar segment composition (PB2, PB1, P3, NP,

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CM1 and CM2, NS1 and NS2) except it only contains one single glycoprotein with hemagglutinin, esterase, and fusion functions (HEF) [9].

Aquatic birds are thought to be the primordial origin for all influenza A viruses. Influenza A is subtyped by identifying the HA and NA segments. Currently, 16 HA and 9 NA distinct segments have been found in avian influenza A viruses detected in wild birds and poultry. This could possibly create 144 HA/NA subtype combinations, but only 55 of these HA/NA subtype combinations were detected in a large survey of 36,809 samples from wild birds [1, 10, 11]. Recently, a new strain of influenza A virus was isolated from little yellow-shouldered bats captured in Guatemala which was significantly divergent from known influenza A viruses subtype. The HA of the bat virus was designated as H17 and the NA as N10, which increase the total number of HA and NA segments [12]. These different HA and NA types provide the potential for a virus bearing new HA or NA by genetic reassortment to emerge. These new subtypes have a greater likelihood of evading the hosts' immunity due to the naivety of the host immune system (antigenic shifts) and can cause pandemic episodes such as the 1918 event. In addition, the accumulation of mutations in two major antigenic glycoproteins, HA and NA, of influenza A viruses constantly creates new variant strains that elude the host immune response (antigenic drifts) and are the primary cause of the regular yearly influenza outbreak [1]. Interestingly, analysis of the evolution of influenza viruses found that the rate of mutation is host dependent with a much higher rate for viruses in mammalian hosts than in birds [13]. In addition to the basic understanding of the viruses (morphology, replication, genome composition, and organization), a myriad of studies in vaccine development, treatments and prevention controls, immune response, infection modeling, pathogenesis, transmission, and epidemiology have been published and extensively reviewed [14–26]. Despite extensive research and surveillance efforts, influenza viruses continue to outwit our efforts to control the disease by undergoing antigenic drifts or antigenic shifts that enable them to escape from preexisting immunity and continue to be a major cause of respiratory tract infection, resulting in significant morbidity and mortality each year.

The primary mitigation treatment for influenza outbreaks is the development of yearly vaccine mixes targeted for the most prevalent virus strains. Consequently, the focus of many detection technologies has evolved toward accurate identification of subtype/strain and understanding of the evolution and molecular determinants of novel and pathogenic forms of influenza that can provide important information for prevention, containment, patient management, and control of influenza infection. Furthermore, the recent availability of potential anti-influenza treatments are only effective if rapid and accurate diagnostic tests for influenza epidemic management are available because the maximal efficacy of such treatment must be started within 36–48 h of onset of symptoms [27–29]. The requirements for optimal treatment also lead to the development of numerous rapid diagnostic tests for influenza viruses. This review discusses the progression of detection methodologies and specific gene targets of current and emerging technologies for detection and strain identification of influenza virus infection as well as their implications in patient management; that is, how they improve diagnosis, treatment, surveillance, and prevention of influenza infection.

2 History of Laboratory Diagnosis of Influenza

The first human influenza A virus was isolated in 1933 using primary chick embryo culture, which revolutionized influenza viruses studies. To date, cultivating influenza viruses in eggs remains the modality for vaccine production [1, 3, 30]. Subsequent to the first studies in eggs, tissue-culture systems using canine kidney cells [Madin–Darby canine kidney (MDCK) cell line] were also developed for virus isolation. Historically, laboratory diagnosis of influenza viruses was viewed as of limited value for disease management because of the long turnaround times of viral culture, limited test sensitivity, and lack of antiviral treatment options [31]. However, differential diagnosis of influenza infection based on clinical signs and symptoms alone is insufficient to provide accurate diagnosis because similar clinical manifestations can also be caused by other respiratory tract viral pathogens, such as respiratory syncytial virus, parainfluenza viruses, coronaviruses, and rhinoviruses, etc.

Laboratory diagnosis of influenza viruses is based on two basic approaches: detection of the presence of the virus, or detection of a host immune response. In addition to viral culture, hemagglutinin inhibition assays (HAI), first described in 1942, were developed for the direct detection of the influenza virus. HAI is based on the principle that the HA on the virus surface can cause agglutination in the presence of erythrocytes. The HA subtype can be determined if agglutination is inhibited when the specimen is mixed with an antibody for a known HA subtype and red blood cells [32]. Besides being used to directly detect virus in a sample, an HAI assay can also be used to detect a host immune response depending on the format of the assay. Other approaches used that probe the host immune response to determine if an influenza virus infection has occurred are complement fixation assays (CF) and enzyme immunoassays (EIAs). CF assays, first developed in 1909 for syphilis diagnosis and then perfected in subsequent

decades for general use in virology, use the properties of the innate immune system in which the complement in the serum interacts with antigen–antibody complexes and does not interact with sensitized red blood cells, which remain unlysed [33]. CF assays will report more accurately for patients that have received an influenza vaccine and have developed antibodies against envelope proteins, which renders HAI tests unreliable for detecting influenza infection [34–36]. EIAs first developed in the 1960s are versatile and widely used methods for virus detection based on the interaction of antigen–antibody, which has also been commonly applied for influenza detection. For this type of method, two or more antibodies (capture and detector antibodies) are used to interact with viral antigen and the detector antibody is labeled with enzyme or interacts with enzyme-labeled third antibodies that react with enzyme subtrate to produce colorimetric changes [35, 37, 38]. Although originally developed to probe the host immune response, this type of test has been developed for the direct detection of influenza virus.

As mentioned earlier, subtyping of influenza A is by HA and NA; the neuraminidase inhibition (NAI) assay, first developed in 1963, was used for detection and typing of NA. This assay is based on the principle that neuraminidase converts fetuin to *N*-acetyl neuraminic acid (5-acetamido-3,5-dideoxy-*N*-acetyl-D-glycero-D-galacto-nonulosonic acid; NANA). NANA converts to β-formylpyruvic acid by periodate oxidation, which then forms a pink chromophore when reacted with thiobarbituric acid. The presence of specific NA antibodies inhibits the occurrence of the reaction, and the specific NA type can be determined [39–41]. This method is rarely used since the development of the last group of detection methods (see below). While subtyping was initially developed to understand the distribution and composition of influenza strains, its current use is focused on monitoring and tracking high-risk strains such as H5N1 and H7N9 infections in domesticated birds and humans.

The latest addition to mature methods for directly testing for the presence of influenza viruses became possible with the development of polymerase chain reaction (PCR) methodology in 1983 [42]. The methodology allows for the amplification of specific nucleic acid sequences to levels that can be detected by various methods. Nucleic acid amplification techniques (NAATs) have been widely adapted in diagnosis of infectious microorganisms including influenza viruses and are capable of targeting a variety of genes and can provide typing information.

3 Current Detection Technologies

There are several diagnostic tests currently available for influenza virus (influenza A and B) detection, including the above-mentioned viral culture and serologic tests, as well as immunofluorescence staining and nucleic acid-based tests (Table 1). The primary focus of the majority of diagnostic tests has been on serving biosurveillance, vaccine development, and pure research needs. For these applications, the tests are optimized for accuracy and sensitivity, but may take significant time and/or be expensive. Some tests have been developed for clinical or epidemic outbreak use and are discussed as a group as rapid diagnostic tests. While all the methods are capable of detecting influenza, they are not all optimal for all usages (best uses noted in Table 1).

Conventional viral culture remains the "gold standard" to which other methods are compared for accuracy but because of its long turnaround time (3–10 days) is not applicable when results are required rapidly. It remains a robust methodology that, besides detecting the presence of influenza and being essential for antiviral susceptibility testing, acts as a critical initial step when gathering specific information regarding circulating strains and subtypes of influenza viruses. Such information is needed to determine the genetic evolution of influenza viruses and guides decisions regarding treatment and chemoprophylaxis, and to formulate vaccines for the coming year [43]. Advances in viral culture techniques such as introducing new cell lines and shell-vial culture methods have improved the detection sensitivity and significantly decreased the turnaround time. The shell-vial culture method (turnaround time 1–3 days) is also versatile in detecting influenza viruses and other respiratory viruses causing similar symptoms, which increases the clinical utilities; thus, it is recommend by the United States Centers for Disease Control and Prevention (CDC) as a laboratory diagnosis procedure [43, 44].

Because individuals are repeatedly exposed to different strains of influenza virus over a lifetime, serologic tests usually require comparison of the antibody titer during convalescent phase with a sample collected during an acute phase. Furthermore, seroconversion usually occurs several days postsymptom onset (appears after ~2 weeks and peaks at 4–7 weeks after infection); thus, serological tests cannot provide results soon enough to guide clinical decisions and are not recommended for routine diagnosis. Although of no direct significance in patient management, serology tests are valuable in conducting seroepidemiological studies to assess the geographic extent, epidemiologic spectrum, and preexisting cross-reactive immunity of the infection [37, 38, 43, 45, 46].

Immunofluorescence staining by direct (DFA) or indirect fluorescent antibody staining (IFA) of clinical specimens, mainly using either monoclonal or polyclonal antibodies against NP and/or M proteins (as well as HA for influenza B viruses), is a relatively rapid technique for

Table 1 Current detection technologies based on assay types

Technology	Targets	Best uses	References
Viral culture	Viral particle	Surv. Vacc. Res.	[1, 43, 44]
Rapid viral culture	Viral particle	Surv. Vacc. Res.	[44]
Immunofluorescence staining: DFA/IFA	NP, M, NS1	Surv. Res.	[47, 48, 90, 200]
Rapid diagnosis tests	NP	Clin.	[43, 143, 144]
Serologic tests			
HAI	HA	Surv. Res.	[32]
CF	NP	Surv. Res.	[34, 35, 38]
EIA	NP	Surv. Res.	[34, 35]
Nucleic acid	M, NP, NS, HA, NA	Surv. Vacc. Res.	[58, 68, 69]
Amplification techniques			

DFA direct fluorescent antibody, *IFA* indirect fluorescent antibody, *HAI* hemagglutinin inhibition, *CF* complement fixation, *EIA* enzyme immunoassay, *HA* hemagglutinin, *M* matrix, *NA* neuraminidase, *NP* nucleoprotein, *Surv.* surveillance, *Vacc.* vaccine selection, *Res.* research, *Clin.* clinical

detecting influenza viruses (1–4 h). The antibodies are labeled with fluorescent dye or interact with second antibodies labeled with fluorescent dye, and the reactions are visualized under a fluorescence microscope. The sensitivity of these assays range from 40–100 % with specificity of 80–100 % compared to gold standard methods. Due to the complexity of the assays, the variations in detection sensitivity and specificity are probably caused by differences in technical expertise, specimen type, and quality (the presence of an adequate number of infected cells), as well as patient selection [27, 47, 48]. Even with the variation in detection sensitivity and their relatively extensive infrastructure requirements, the immunofluorescence staining methods have demonstrated high accuracy in detecting influenza virus and have been adapted for identification of specific influenza subtypes using monoclonal antibodies specific for HA subtypes, such as H1, H3, H5, and H7 [49–51]. DFA and IFA are valuable in confirming rapid diagnostic testing results [31, 52–54].

NAATs have gained a more prominent role in laboratory diagnosis of influenza because of superior sensitivity and specificity of the assays, rapid turnaround time, and compatibility with automation. The basic principles of NAATs are explained in detail in various reviews [55–59]. The following section reviews currently available NAATs for influenza diagnosis, their specific gene targets, and their implications in patient management.

Reverse transcription PCR (RT-PCR) and real-time RT-PCR are the most common NAATs used for influenza virus diagnosis. A simple search will turn up thousands of publications in PubMed on methods for detecting influenza virus using PCR-based methods. These methods can be used in any properly equipped research facility but depend on the individual laboratory obtaining the required reagents. While the sensitivity of all of these methods is usually very good, the specificity can vary widely depending on the specific sequence chosen as the target by the researchers. Because several different respiratory pathogens can cause symptoms similar to those of influenza, quite a few multiplex RT-PCR methods that target several gene targets at once have been developed for encompassing respiratory pathogens including influenza viruses. The coverage of the multiplex RT-PCR methods varies from only influenza viruses (different strains of influenza A and influenza B viruses or different type of influenza A viruses) to several different respiratory pathogens [60–66]. Real-time RT-PCR methods use fluorescence-labeled probes or fluorescent dyes to detect the PCR products without postamplification manipulation and added detection sensitivity, which further advances the utility of PCR-based methods for influenza detection. Indeed, the first commercially available kit for influenza was Hexaplex assay by Prodesse, Inc. (now Hologic Gen-Probe Inc., San Diego, CA). The Hexaplex assay is multiplex RT-PCR with postamplification hybridization with product specific probes, which is quite time consuming. The assay was later replaced by Pro-Flu+, which is a real-time multiplex RT-PCR kit (Pro-Flu+ targets M gene of influenza A viruses, NS1 and NS2 genes of influenza B viruses) [67]. Most of these PCR-based assays use the NP and M genes for type-specific detection of influenza viruses because these are highly conserved, while genes encoding surface antigens HA and NA are targeted for subtyping influenza A viruses [58, 67]. NS gene is also used in some of the assays for type-specific detection [68–71]. RT-PCR, multiplex RT-PCR, and real-time RT-PCR represent important tools for the timely and sensitive diagnosis of influenza A virus infections with turnaround times of 1–4 h; however, these assays require laboratory set up, experienced personnel, and are not suitable for point-of-care diagnosis, which reduces their effectiveness in immediate clinical management.

RT-PCR-enzyme immunoassay (PCR-EIA or PCR-ELISA) and PCR-restriction fragment length polymorphisms (PCR-RFLP) are derivatives of RT-PCR methods. PCR-EIA/PCR-ELISA use product-specific probes to capture PCR products or labeled primers that can be captured by covalent binding (i.e., biotin–streptavidin interaction). Most of these assays target NP gene for detection, with a few assays targeting NS and M genes, while HA genes are used for subtyping influenza A viruses [60, 72–78]. Although PCR-EIA/PCR-ELISA are more sensitive than conventional PCR-based assays, these methods require postamplification manipulation with longer turnaround times and require special laboratory set up, which may be useful for screening a large number of samples. PCR-RFLP uses the electrophoresis profiles of enzyme-digested PCR products to differentiate different strains of influenza viruses (genotyping) or discern drug-resistance markers. All eight gene segments have been targeted for PCR-RFLP analysis and the most common ones are HA, NA, and M genes [79–95]. These techniques also require postamplification processing, which is time consuming and labor intensive, and thus are more suitable for epidemic surveillance, but have no immediate impact in patient treatment.

In addition to PCR-based methods, there are several isothermal nucleic acid amplification methods developed for pathogen detection including nucleic acid sequence-based amplification (NASBA), loop-mediated isothermal amplification (LAMP), rolling circle amplification (RCA), exponential amplification reaction (EXPAR), isothermal and chimeric primer-initiated amplification of nucleic acids (ICANs), signal-mediated amplification of RNA technology (SMART), helicase-dependent amplification (HDA), transcription-mediated amplification (TMA), and strand displacement amplification (SDA) [96–106]. The basic principles of these methods have been reviewed elsewhere [55, 57, 59]. The most common isothermal amplification methods used for influenza detection are NASBA and LAMP, and these are described below in detail.

NASBA is an RNA-based nucleic acid amplification technique that was developed in 1991. It uses T7 RNA polymerase, RNaseH, and reverse transcriptase with 5′ primer containing the promoter sequence for bacteriophage T7 DNA-dependent RNA polymerase and 3′ primers specific for target DNA containing complementary sequence to detecting probes. The detecting probes can be labeled with either ruthenium-based electrochemiluminescent (ECL) tag, or biotin, or fluorescence. Depending on the probes used, the amplified products can be detected by electrochemiluminescence or ELISA, or fluorescence (real-time NASBA) [96, 107–109]. The main gene target for influenza A detection is HA gene while PA gene is targeted for influenza B detection [108, 110–117]. One multiplex NASBA was developed for influenza A and B, human parainfluenza virus 1–4, respiratory syncytial virus, rubella virus, and coxsackie virus, which uses M gene for influenza A and NS1 gene for influenza B detection [118]. Similar to RT-PCR, NASBA methods have high sensitivity, specificity, and similar turnaround time; however, these methods do not require thermocycling, have less stringent instrument requirements, and can be very useful for high-throughput application. In addition, real-time NASBA can be adapted and developed toward potential point-of-care assays for influenza detection that will have an impact on clinical management.

Loop-mediated isothermal amplification (LAMP) method was developed by Notomi et al. in 2000. It uses multiple primers and DNA polymerase with strand displacement activity in reactions performed under isothermal conditions. These methods have high sensitivity, specificity, yields products in a short time frame (30–90 min), and the products can be viewed via turbidity or fluorescence, which simplifies the instrumentation requirements for the assay [97, 119]. The majority of the reverse transcription LAMP (RT-LAMP) methods are developed for avian, equine, and swine influenza detection, and relatively fewer assays for human influenza detection. For RT-LAMP methods, the HA gene is the main target for influenza A virus detection, and NP gene for influenza B virus detection [120–142]. The major limitation of RT-LAMP is the complexity of primer design, making it generally not suitable for large-scale multiplex reaction. However, the robustness, simplicity of the reaction setup, and minimal instrumentation requirements make RT-LAMP a potential point-of-care assay for influenza detection.

Tests for influenza detection that are of more immediate clinical relevance are rapid diagnostic tests. Rapid diagnostic tests are based on immunochromatographic lateral flow or membrane-based immunoassays that provide results in 30 min or less. They have been specifically designed for point-of-care screening. There are several commercially available tests, and most of the assays target influenza viral nucleoprotein, which can detect as well as distinguish influenza A and B (Table 2 lists the characteristics of 25 rapid diagnostic kits). None of these tests provide subtype information on influenza A viruses and the sensitivities of these tests are approximately 50–70 % with 90–95 % specificity when compared to the gold standard of viral culturing [27, 54, 143, 144]. Despite their low sensitivity, rapid diagnostic tests can be useful in patient and outbreak management, especially when other tests are not readily available [31, 43, 143, 144].

Table 2 Summary of commercially available rapid influenza diagnostic tests[a]

Name	Approved specimen	Assay time (min)	Manufacturer
3M™ rapid Detection Flu A + B Test	NP swab/aspirate, NW, NA	15	3M
BD Veritor System for Rapid Detection of Flu A + B	NP swab, NS	10	Becton Dickinson
Directigen EZ Flu A and B	NP swab/aspirate/wash, TS, BAL	15	
BinaxNOW® Influenza A and B	NP swab/aspirate/wash, NS	15	Alere
Clearview® Exact Influenza A and B	NS	15	
BioSign® Flu A + B	NP swab/aspirate/wash, NS	15	Princeton BioMedtech
OSOM® Influenza A and B	NS	10	Genzyme
QuickVue® Influenza Test[b]	NA, NS, NW	10	Quidel
QuickVue® Influenza A + B Test	NP swab, NA, NS, NW	10	
Sofia Influenza A + B	NP swab/aspirate/wash, NW	15	
SAS™ FluAlert A and B	NA, NW	15	SA Scientific
SAS™ FluAlert A[b]	NA, NW	15	
SAS™ FluAlert B[b]	NA, NW	15	
TRU FLU®	NP aspirate/swab, NW	15	Meridian Bioscience
XPECT™ Flu A and B	NS, NW, TS	15	Remel/Thermo Fisher
TRU FLU	NP swab/aspirate, NS, NW	15	Meridian Bioscience, Inc.
FLU OIA	NP aspirate/swab, TS, Sputum	15	Biostar
FLU-A-Dot-ELISA[b]	NP swab, NS	20–30	Beijing Wantai Biological Pharmacy Enterprise Co.
SD Biolone influenza Ag A/B/A(H1N1)Pandemic	NP aspirate/swab, NS, TS, NA	10–15	SD Biolone
ESPLINE Influenza A and B-N	NP swab, NS, NA	15	Fujirebio Inc.
Actim influenza A and B	NP swab, NS	10–15	Medix Biochemica
ZstatFlu	TS	20	ZymeTx, Inc.
NanoSign Influenza A/B	NS, TS		SICL Co.
OSOM Influenza A and B	NS	10	Sekisui diagnostics
Influenza A/B antigen test	NS, TS	10–15	Humasis

NA Nasal aspirate, *NP* nasopharyngeal, *NW* nasal wash, *NS* nasal swab, *TS* throat swab, *BAL* bronchoalveolar lavage

[a] Based on recommendations from the CDC web site [43]

[b] Test does not distinguish influenza A and B virus

4 Emerging Multiplex Detection Technologies for Detection and Strain Identification

Multiplex methods provide the capability to simultaneously test several conditions to gain either greater typing information and/or test for several respiratory pathogens, including influenza viruses. Because the clinical presentation of influenza particularly in its early stages is so similar to several other respiratory pathogens, it is recognized that such methods are required to maximize the efficacy of relatively new therapeutic approaches using antiviral medicines. These methods face a more complicated path to FDA approval as diagnostics and so many are still in development and in research use only.

RT-PCR methods can be multiplexed, but the maximum number of detectable targets in a single assay is limited by capabilities of parsing out PCR products. For example, the number of detectable targets of real-time PCR is limited by the machine used to monitor the reaction, usually four targets due to cost considerations. This method monitors the fluorescence of different dyes, which must have sufficient separation in their emission peaks to distinguish them, and tracks the individual multiplex targets. For broader range detection, several emerging techniques based on multiplex PCR have been developed for respiratory pathogen detection, such as xTag® respiratory virus panel, ResPlex II™ panel v2.0, Multicode-PLx (EraGen Biosciences), RespiFinder assays, FilmArray Respiratory Panel, and others. All these assays require special instruments and are suitable for clinical laboratory testing but not for point-of-care diagnostics.

The xTag® respiratory virus panel (RVP) and RVP FAST were developed by Luminex Corporation (Austin, TX, USA) to detect influenza A (including subtype H1 and

H3), influenza B, respiratory syncytial Virus (RSV), human metapneumovirus (hMPV), rhinovirus (RhV), and adenovirus (AdV) using fluorescent beads with specific antitag sequences that hybridized with specific biotin-labeled PCR products. For influenza A viruses, the gene targets used for testing are M genes for general detection and HA for subtyping H1, and H3, while *prehemagglutinin* gene is targeted for influenza B virus detection. The turnaround times for the assays are approximately 6–24 h, which has great utility in hospitalized patient management, and could potentially affect clinical management [145, 146]. Similarly, Multicode PLx RVP developed by EraGen Biosciences (recently acquired by Luminex and this product is discontinued) uses M genes as targets for influenza detection [147]. Based on a similar principle, Resplex II^{TM} panel v2.0 was developed by Qiagen (Valencia, CA, USA) to detect influenza A and B viruses, RSV A and B, parainfluenza (PIV) 1–4, hMPV, RhV, AdV B and E, Coxsackieviruses/Echoviruses, Coronaviruses (CoV, strains NL63, HKU1, 229E and OC43), and Bocavirus (BoCV). The proprietary nature of the primers and probes makes it impossible to determine the gene targets [148].

RespiFinder® assays (RespiFinder® 15, 19, 22, and Smart 22) are based on the multiplex ligation-dependent probe amplification (MLPA) technology and developed by Patho Finder B. V. (The Netherlands) using M gene as the target for both influenza A and B viruses. The turnaround times for these assays are approximately 8 h, which has great utility in inpatient management, and could potentially affect clinical management [149, 150]. The FilmArray respiratory panel developed by Idaho Technology Inc. (now BioFire Diagnostics Inc., Salt Lake City, UT, USA) is based on multiplex real-time RT-PCR coupled with amplicon melt curve analysis for pathogen detection. For influenza A viruses, M, NS1, and HA genes are selected targets for detection, and HA gene is used for influenza B [151]. The FilmArray system is an automated system, including sample preparation to nucleic acid amplification, and requires very little hands-on time with turnaround times at approximately 1 h. While it is easy to use and provides rapid results, the low throughput and qualitative results may hinder its application in laboratories receiving large numbers of samples and in some clinical settings.

The INFINITI® plus analyzer (AutoGenomics, Vista, CA) is an automated platform that couples multiplex PCR with microarray hybridization. The INFINITI® Respiratory Viral Panel Plus is designed to detect 25 common respiratory viruses, including influenza A and B, PIV 1–4, RhV A and B, Enterovirus (EV) A–D, CoV (HKU1, OC43, NL63, and 229E), hMPV A and B, RSV A and B, AdV A–C and E. For influenza A and B, NP gene is targeted for detection and the turnaround time is approximately 7 h. This assay is designed for clinical laboratory and not for near-patient testing [152]. The INFINITI® FLU A-sH1N1 is designed to detect influenza A and differentiate between influenza A and subtype swine H1N1 only.

The padlock probes technique developed in 1994 by Nilsson et al. [153] uses two target-complementary oligonucleotides to hybridize with target sequence. Once hybridized to the target sequence, the probes can be linked through ligation and the reacted probes can be detected through a linker or amplified through rolling circle amplification. This unique technology has been applied for genotyping all HA and NA subtypes of avian influenza viruses [153–155].

RT-PCR/electrospray ionization mass spectrometry (RT-PCR/ESI-MS) uses ESI-MS to analyze the base composition of PCR products. Based on this principle, PLEX-ID/Flu assay was developed for detecting and typing influenza viruses. All eight gene segments of influenza A virus and three gene targets (PB2, HA, NA) of influenza B virus were used in this assay [156–160]. Due to the special instrumentation requirements of this technique, it is more suitable for surveillance purposes.

Multiplex PCR using primers labeled with molecular tags of different molecular weight (mass-code tagging system developed by Qiagen) was also used for respiratory pathogen detection. The microbial identity is determined through analysis of cognate tags of the PCR products. The gene targets for this assay are M, HA (1–3, 5), and NA (1, 2) genes for influenza A viruses, and HA for influenza B viruses [161].

There are several highly multiplex platforms, such as Scalable Target Analysis Routine (STAR) Technology, which may be suitable for respiratory pathogen detection, but have not yet been developed for influenza detection [62].

DNA microarrays contain hundreds to tens of thousands of probes, and a microarray experiment provides massive multiplex detection capabilities that offer potential for simultaneous detection of many pathogens. The concept of using microarrays for broad-spectrum pathogen detection has its obvious appeal and has been explored for use in pathogen diagnostic applications. Various types of microarrays including spotted oligonucleotide microarrays, and high-density resequencing microarrays, have been used in assorted pathogen detection and have been reviewed in detail previously [162–166]. For influenza viruses, there are several different types of microarrays available for respiratory pathogen detection including influenza viruses and most of these arrays have comparable detection sensitivities and specificities to other molecular diagnostic tests. The gene targets of influenza viruses for these assays are mainly HA, NA, and M genes. NP and NS genes were also used in some of the microarrays [167–178]. Microarray technologies require specialized instrumentation and are more suitable for research and surveillance purposes.

Next-generation sequencing (NGS) technologies differ from traditional Sanger chemistry by enzymatically or mechanically breaking down genomic DNA into smaller fragments, then ligating these fragments with adapters for sequence reading during DNA synthesis (sequencing by synthesis). These technologies furnish a large number of short sequence reads (current read length is ~50–500 bp). The principles of NGS and various platforms are explained in detail in various reviews [179–182]. NGS technologies can generate large sequence datasets on a short time scale, which has appeal in pathogen detection/diagnostics, but it still needs sophisticated bioinformatics support to accommodate high-depth datasets. A few studies have reported success in identifying microorganisms from biological samples. These technologies require specialized instrumentation, currently have low detection sensitivity, and have long turnaround time for bioinformatics analysis such that the NGS technologies are more suitable for genomic characterization of novel bacterial and viral pathogens, comparative genomic analysis, and genetic tracking [183–185].

5 Gene Targets of Diagnostics

The traditional targets for identification of influenza viruses have been the NP, M, or NS genes because these provide commonality in identification across different identification methods. All the diagnostic methods mentioned have versions designed to test one or all of these targets. Methods that use antibodies as part of the identification process invariably have cross reactivity so that even if a new strain variant is present, there is still the possibility for detection. For subtyping, HA and NA segments are the targets because these define the subtypes of the viruses and identify highly pathogenic strains; that is, avian H5N1 and H7N9. Newer NAAT methods are much more specific in matching the target and even small changes in sequence can result in a test targeting HA or NA to fail. Consequently, more conserved gene segments have been included in the range of genes targeted. A typical target is the matrix gene segment. Tests for this segment typically report only the presence of type and no subtype information is provided. It is possible to couple tests for this target with tests for the HA and NA, producing a test that will detect influenza with high confidence and will subtype if the strain is of a known type.

Since 1990, two types of anti-influenza drugs, namely, the neuraminidase inhibitors (oseltamivir and zanamivir), and the M2 ion-channel inhibitors (amantadine and rimantadine), have become available for the treatment and chemoprophylaxis of influenza infections [1]. These antiviral drugs are most effective if given early in the infection. Similar to antibiotics resistance that develops in bacterial pathogens, the use of these antiviral drugs has already revealed/induced the existence of resistant strains. For neuraminidase inhibitor, drug-resistance mutations developed to affect NA catalytic sites, which consisted of 8 functional residues (R-118, D-151, R-152, R-224, E-276, R-292, R-371, and Y-406) and surrounded by 11 framework residues (E-119, R-156, W-178, S-179, D-198, I-222, E-227, H-274, E-277, N-294, and E-425) (N2 numbering system). These residues are conserved in all influenza A and B viruses. Several mutations of the catalytic sites and surrounding framework were identified to be associated with reduced sensitivity to neuraminidase inhibitor; however, only H274Y (H275Y in N1 numbering) is unequivocally considered oseltamivir resistant, while I222R, I222K (I223R and I223K in N1 numbering) are associated with zanamivir resistance [186, 187]. For M2 inhibitor, drug resistance is associated with mutations that cause amino acid substitutions of residues 26–34 in the transmembrane domain of the M2 protein, and the predominant mutation that confers M2 inhibitor resistance is S31N [187]. Consequently, the segments that indicate antiviral resistance, the NA and M2 genes, have also become important gene targets as well for single nucleotide polymorphism (SNP)-based assays, such as sequencing (Sanger sequencing or pyrosequencing) and real-time RT-PCR allelic discrimination, to screen drug-resistance mutations.

6 Influenza C Virus

Influenza C virus, in contrast to its infamous cousins influenza A and B viruses, causes mild respiratory tract illness. As a result, it receives much less attention. Serological studies do indicate a wide distribution of influenza C virus and that most people have acquired antibodies to the virus early in life, which undermines its epidemiological and clinical impacts. Consequently, fewer studies provide information regarding the prevalence of active influenza C virus infection in the general population and few detection assays of influenza C viruses are available. While any of the assays already mentioned for influenza A detection could be applied for the detection of influenza C, cell culture and RT-PCR are the most common assays used for influenza C virus isolation and characterization [188–190]. A few multiplex RT-PCR methods developed for encompassing respiratory pathogens also include influenza C virus targeting NP and HEF genes. There is no rapid diagnostic test for influenza C detection [61, 64, 65, 191].

7 Conclusion

The current collection of detection methods for use in surveillance, vaccine selection, and pure research with

regards to accuracy and speed of testing are all very mature and perform quite well for those uses. In this context, these methods do not really have any serious limitations. They only suffer serious limitations if they are applied for purposes other than what they were designed for, such as determining individual patient care. These methods suffer from their cost and/or long turnaround time not justifying the information provided in a clinical setting. For this very reason, rapid diagnostic tests were developed. While these tests are fast and simple, they suffer from a lack of sensitivity so that a positive result can be trusted as accurate but a negative result cannot be used to rule out influenza infection. The other issue for these tests is more a reflection of the context in which people are infected by influenza. While influenza is the major cause of respiratory illness for a portion of each year, there remain several other potential causes that all exhibit similar early symptoms, which is when intervention might be more effective. Consequently, rapid testing in clinical settings is normally applied only when more serious symptoms have already developed. This is because any test that only reports on one of these pathogens does not provide a great deal of aid to treatment practice when a patient only has early symptoms. Information about all possible causes of the early symptoms needs to be provided in the same test before the potential benefits of such tests will make sense in clinical treatment situations.

Methods that test for multiple pathogens simultaneously are the current diagnostic advancement being worked on that may significantly alter clinical treatment procedures. While a few multiple-pathogen methods have been developed, none is truly ready for use in a clinical setting. New technologies that allow the rapid detection of multiple pathogens from multiple markers in a single test will improve diagnostics for a large panel of respiratory pathogens that includes influenza viruses and better direct use of antivirals or antibiotics. Consequently, the gene targets used for detecting influenza viruses in future diagnostic tests are likely to broaden to include targets for typing, subtyping, and antiviral resistance identification in the same test. A key to advancing these tests to routine use in a clinical laboratory would be increased automation of steps within the various approaches. Even with an improved detection test that could be used in clinical settings, the question of whether clinical treatment procedures will actually change is not a foregone conclusion. The therapeutic management options are limited to a few antiviral treatments that need to be immediately administered and already there are indications of strains being resistant to these drugs.

The prospects for the use of NGS is much more difficult to predict with the current technologies. The current machines have been optimized for efficient read generation from single organisms with large genomes. Influenza virus is among one of the smallest genomes for an organism and normal examination of influenza is as a minor component from a sample that contains host genomic material. Until there is a significant change in the NGS technologies, it is unlikely that these methods will find use in the established clinical and surveillance settings. The more promising application of this technology is for new research to lead to a better characterization of rapidly evolving viral quasispecies and understanding of the evolution of the virus within a host. This has been an area of ongoing research with an excellent review on progress up to 2007 [192]. The data accessible from NGS systems has resulted in more recent research focusing on taking advantage of the new information that is available on the phylodynamics to give insight into the generation of new influenza strains. The implications of this understanding may help to direct the search for new antiviral treatments or alter the selection and production of vaccines [193–199].

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