Published in final edited form as:

Expert Rev Anti Infect Ther. 2010 May ; 8(5): 517–527. doi:10.1586/eri.10.24.

Methods for molecular surveillance of influenza

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Abstract

Molecular-based techniques for detecting influenza viruses have become an integral component of human and animal surveillance programs in the last two decades. The recent pandemic of the swine-origin influenza A virus (H1N1) and the continuing circulation of highly pathogenic avian influenza A virus (H5N1) further stress the need for rapid and accurate identification and subtyping of influenza viruses for surveillance, outbreak management, diagnosis and treatment. There has been remarkable progress on the detection and molecular characterization of influenza virus infections in clinical, mammalian, domestic poultry and wild bird samples in recent years. The application of these techniques, including reverse transcriptase-PCR, real-time PCR, microarrays and other nucleic acid sequencing-based amplifications, have greatly enhanced the capability for surveillance and characterization of influenza viruses.

Keywords

influenza A virus; molecular surveillance; real-time PCR; RT-PCR; surveillance

Influenza viruses are a major cause of respiratory tract infections in humans, resulting in significant morbidity, mortality and economic losses. In the USA alone, influenza kills at least 36,000 people in an average year and is responsible for more than 200,000 hospitalizations [201], but in some years can lead to even higher mortality. Novel influenza A viruses are unpredictable and occasionally cause pandemics, including the pandemics of 1918 (an H1N1 subtype virus), 1957 (H2N2) and 1968 (H3N2). The 1918 pandemic virus caused approximately 675,000 deaths in the USA and killed approximately 40 million people worldwide [1]. The highly pathogenic avian influenza (HPAI) virus of the H5N1 subtype has caused major concern for its pandemic potential since this lineage arose from Asia in 1996 [2]. Unexpectedly, however, the first pandemic of the 21st Century was caused by a novel H1N1 influenza A virus that first emerged in March 2009 in Mexico and Southwestern USA [3]. In the ensuing months, it spread globally to become a public health emergency in the international community. This pandemic highlights the urgent need for fast, accurate and sensitive detection of influenza A viruses and their rapid subtyping within surveillance and diagnostic networks.

Influenza viruses are single-stranded, negative-sense RNA viruses of the family *Orthomyxoviridae*. They are divided into three types: A, B and C; types B and C are generally

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Financial & competing interests disclosure

This research was supported by the Intramural Research Program of the NIAID and the NIH. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

only found in humans. Type A influenza viruses are responsible for most seasonal influenza epidemic morbidity and mortality, and all influenza pandemic strains. Type A is further divided into subtypes by antigenic characterization of the two surface glycoproteins: hemagglutinin (HA) and neuraminidase (NA). Influenza A virus contains eight gene segments and encodes 10–11 proteins, including HA, NA, matrix proteins (M1 and M2), nucleoprotein (NP), nonstructural proteins (NS1 and NS2) and a polymerase complex (PA, PB1, PB2 and PB1F2). Based on the antigenic characterization of the HA and NA, the strain can be classified into sixteen HA and nine NA subtypes [4,5]. Aquatic birds are the natural reservoirs of influenza A viruses and all sixteen HA and all nine NA subtypes in most of the possible combinations have been isolated from these avian species. Only a small subset of influenza A virus subtypes has become established in mammalian species, including humans [6]. Influenza A viruses evolve rapidly due to both antigenic drift and reassortment (antigenic shift), and in humans, new antigenic variants emerge constantly to give rise to yearly epidemics [7].

When an antigenically novel strain emerges and circulates widely in people it can cause a pandemic. The 2009 pandemic swine-origin H1N1 has been demonstrated to have emerged by reassortment between two established swine influenza virus lineages [3,8]. Even though it is too early to predict the impact of this pandemic or how the virus will evolve, this novel virus has already spread globally and rapidly [9].

Fast, accurate and comprehensive methods for diagnosis of influenza A virus infection are needed for surveillance of emerging viruses, for outbreak management, as well as for early antiviral treatment, prophylaxis and infection control. The traditional ‘gold standard’ methods for typing and subtyping influenza A virus have been virus isolation followed by HA and NA subtyping using antigenic assays [10]. Serological analysis can also provide accurate information of influenza virus infection for surveillance but it is a retrospective method because both acute and convalescent serum is required. Both of these methods cannot, in practice, be used for identification of influenza viruses of novel subtypes owing to the lack of available serologic reagents. Growing virus in embryonated chicken eggs and/or tissue culture can generate a large quantity of virus for further characterization, but it is time-consuming and labor-intensive. It normally requires 3–7 days to grow a virus prior to antigenic tests and certain conditions for specimen storage and transport are needed. Other influenza A virus tests such as antigen-based assays can provide rapid detection for clinical diagnosis and surveillance, with results in approximately 30 min, but these assays have a relatively low sensitivity and high requirements for sample quality. Some antigen tests, such as rapid influenza diagnostic tests (RIDTs), can detect influenza A and/or influenza B but cannot distinguish influenza A subtypes. With all the limitations of these traditional methods, molecular diagnostic tests have proven themselves to be the most invaluable tools in the identification and control of influenza A virus infection. The applications of PCR-based techniques, microarray and other molecular sequencing and subtyping methods have expanded exponentially in recent years. They have rapidly transitioned from basic research to detection formats and are now widely employed in both surveillance and diagnosis for their rapid, reliable and inexpensive features.

PCR-based methods

Conventional PCR/reverse transcriptase-PCR

The development of reverse transcriptase-PCR (RT-PCR) techniques has enabled the rapid and accurate diagnosis of influenza A virus infection through sensitive detection of specific viral genomic nucleic acid sequences. Most RT-PCR techniques are performed either by one-step or two-step RT-PCR. One step RT-PCR combines the reverse transcription and PCR together using one of the PCR primers, oligo-(dT)-primers or random primers for reverse transcription, while two-step PCR is carried out by performing reverse transcription first followed by PCR. In these procedures, purified influenza viral RNA is first reverse transcribed

into cDNA, the cDNA then amplified with specific primers. Based on the conservation of the first 12 and 13 nucleotides at the 3' and 5' terminus, respectively, in all eight gene segments in influenza A virus, a 'multi-segment RT-PCR' approach was developed [11,12]. This robust method can simultaneously amplify genomic RNA segments directly from human nasopharyngeal and oropharyngeal swab specimens regardless of virus subtype [12]. This complete genome amplification of influenza A virus can overcome the difficulties of analyzing some viruses that are not easily recovered by conventional virus isolation, and provide accurate virus subtype information with subsequent sequencing analysis.

Molecular subtyping of an unknown influenza A virus is difficult owing to the highly variable sequences of the 16 different HA and nine different NA gene segments [13]. Degenerate RT-PCR is a very powerful tool for finding new genes or gene families. By aligning the sequences from a number of related genes, the conserved sequences can be used for designing degenerate primers. Owing to the need to rapidly identify influenza subtypes, efforts for detecting multiple HA subtypes simultaneously have been made by many groups. Gall *et al.* developed a HA detection method called PanHA RT-PCR. A mixture of five degenerate primers is used in this method to amplify a fragment of 164–176 bp spanning the HA₀ cleavage site of influenza A virus of all 16 HA subtypes [14]. This PCR product can be directly sequenced for molecular characterization of the HA₀ cleavage site sequences and allows both molecular pathotyping of H5 and H7 and subtyping of non-H5 or -H7 within a single reaction. In a surveillance study of avian influenza A virus in Alaska wild birds, Wang *et al.* designed four pairs of degenerate primers to amplify all known avian influenza A virus HA subtypes directly from ethanol-fixed cloacal swab material [15]. Using these degenerate primers, they were able to accurately subtype viruses present, and they were also able to detect mixed infections. Processing swab specimens in ethanol at room temperature not only reduces the cost for storage and transportation but is also time-saving. This method for HA subtyping thus demonstrated an easy and inexpensive technique for field avian influenza A virus surveillance.

Degenerate RT-PCR can also contribute to the subtyping of influenza A virus NA genes. Most published RT-PCR strategies for typing and subtyping influenza A viruses are based on the matrix and HA segments respectively, but some methods have recently been developed for NA gene subtyping. Alvarez *et al.* designed a pair of degenerate primers with M13 tags for amplifying all nine NA subtypes [16]. In this study, a band of approximately 253 bp was clearly visible with 40 femtogram of starting RNA using 36 cycles, and all bands visualized in agarose gels were adequate for direct sequencing. This method provides the possibility of high-throughput screening for influenza A virus NA gene subtyping. In another recent study, nine pairs of degenerate subtype-specific primers were designed based on an analysis of 509 NA gene sequences available in GenBank [17]. By running nine RT-PCRs simultaneously in a set of separate tubes, the subtype of NA can be simply determined by subsequent agarose gel electrophoresis and ethidium bromide staining [17].

Real-time PCR/RT-PCR

Real-time PCR was a key development in PCR-based technology. This technique uses chemistry and instrumentation platforms to amplify and simultaneously quantify a targeted DNA molecule. Currently, four different techniques are in general use: TaqMan[®], Molecular Beacons, Scorpions[®] and SYBR[®] Green for real-time PCR. All of these chemistries allow detection of PCR products via the generation of a fluorescent signal. Real-time PCR and real-time RT-PCR (RRT-PCR) are now widely and increasingly used in influenza A virus detection, diagnosis and surveillance because of their high sensitivity, good reproducibility and wide dynamic quantification range with potential for high-throughput screening of a large number of samples. In the past decade, real-time PCR has become the most useful tool for avian influenza virus (AIV) detection for routine surveillance, outbreak and research [18,19].

Most of the real-time PCR assays used in influenza A virus surveillance and diagnosis are based on a TaqMan approach, in which a probe is designed to hybridize to an internal region of the PCR product so that the highest sensitivity and specificity can be achieved during the PCR amplification. Because of the conserved nature of the matrix gene segment among different type A influenza viruses, specific matrix gene primers and probes have often been designed for influenza virus typing (i.e., distinguishing type A and type B influenza virus) in human samples. Another use of matrix gene-based real-time PCR is as a screen for type A influenza virus in samples from humans or animals. This assay is especially important when negative results are found for HA or NA detection. In a large field surveillance study in wild ducks in Alaska, Runstadler *et al.* used an influenza A virus matrix gene-specific TaqMan probe/primer set to screen cloacal swab samples and found 25.6% influenza A virus-positive samples in these wild duck populations [20]. In another study, a TaqMan assay with a minor groove binder (MGB) probe was designed based on the sequence homology of matrix genes among different subtypes of influenza A strains of avian, human, swine and equine origin [21]. This assay is extremely sensitive, and the minimum detectable amount of avian influenza A reference virus corresponded to 0.001 TCID₅₀/reaction and 0.08 EID₅₀/reaction, respectively. RRT-PCR has also been used for AIV surveillance for the prevention and control of low pathogenicity H5 and H7 avian influenza in live-bird markets in the USA [22].

The Asian-origin HPAI H5N1 has caused infections in both wild and domestic avian species, humans and other mammals since 2003 [23], and a number of TaqMan RRT-PCR methods specific for detecting the HA, NA and matrix genes of highly pathogenic (HPAI) H5N1 AIVs were developed [24–27]. For investigating viral replication in cardiac and skeletal muscle of chickens after experimental intranasal inoculation with the HPAI H5N1 virus, RRT-PCR was used for detection of H5N1 in chicken meat [28]. In 2007, Chen *et al.* also described a sensitive and specific RRT-PCR method for the detection of influenza A subtype H5N1 [26]. Recently, a TaqMan assay was designed by using two sets of primers and probes to amplify two different regions of the HA gene of HPAI H5N1 virus [29]. Their results showed that the assay is specific for the H5 subtype and capable of detecting H5 RNA samples obtained from infected birds. A similar method was also previously used to detect and quantify HPAI H5 RNA in clinical samples obtained from H5N1-infected patients [30].

With the emergence of the 2009 pandemic influenza A H1N1 virus, real-time PCR methods rapidly became the primary assay for diagnosis and surveillance. The CDC developed a real-time PCR protocol for 2009 H1N1 influenza A virus detection shortly after the emergence of the outbreak. A one-step RRT-PCR approach, which targets the matrix gene of the novel influenza A/H1N1, was designed and successfully used to detect novel H1N1 in clinical specimens and did not cross-react with seasonal influenza A, subtypes H1N1 and H3N2 viruses and swine influenza A (H1N1) [31]. Two TaqMan probes targeting the HA and NA genes were also developed for detection of the novel influenza H1N1, seasonal H1N1 and H3N2 using human respiratory samples [32]. In a very recent study, several fluorescence-labeled locked nucleic acid hydrolysis probes from Universal Probe Library were chosen and evaluated for detection of HA and NA segments of pandemic influenza A (H1N1) using real-time PCR reactions. The analytical sensitivities and specificities of the assays were equivalent to a reference assay recommended by the German health authorities [33].

Although generally of high sensitivity and specificity, TaqMan probes needed for each PCR target are relatively expensive for large-scale screening and surveillance in many applications. SYBR Green chemistry is an alternative method that has been used to perform real-time PCR analysis. Since the dye of SYBR Green binds to dsDNA, there is no need to design a probe for any particular target being analyzed. In some respects, SYBR Green-based methods provide the simplest and most economical choice for influenza A virus screening even though an

extensive optimization is usually required to enhance the sensitivity and specificity of the reactions.

Since SYBR Green melting curve analysis can be used to identify and map single nucleotide polymorphisms [34], some research groups are now using SYBR Green based real-time PCR to detect, quantitate and distinguish influenza A virus. In 2005, a study of ethanol-fixed nasopharyngeal swab samples by Krafft *et al.* demonstrated that the human influenza A virus H1N1 and H3N2 subtypes co-circulating in 2002–2003 could be distinguished by melting temperature (T_m) analysis based on the minor sequence variation in different matrix gene targets [35]. The estimated amplification limit of this SYBR Green assay was 390 femtogram of template RNA (approximately 50,000 viral copies). In a more recent report, a SYBR Green one-step real-time PCR was used to amplify matrix genes and was able to distinguish the seasonal influenza H3N2 and the newly emerging H1N1 viruses based on their dissociation curves [36]. The sensitivity between SYBR Green real-time PCR and TaqMan RRT-PCR was investigated previously in a screening system developed for monitoring influenza A virus in wild birds [37]. In this study, two SYBR Green reactions and one TaqMan reaction were performed and compared for each of the samples. The result showed that the two SYBR Green assays had a better performance regarding the number of detected influenza A samples as compared with the PCR reaction using a specific probe. In a more recent study, a SYBR Green I RRT-PCR assay was also designed to detect and differentiate influenza A H1N1 virus in swine populations [38].

Multiplex PCR

Multiplex PCR is a variant PCR that simultaneously amplifies multiple DNA targets using more than one pair of primers in a single reaction tube. Combined with conventional RT-PCR or real-time PCR, multiplex PCR has been successfully used in detection, typing and subtyping of influenza A virus and can provide accurate, sensitive detection for influenza virus surveillance. Most of the developed methods for multiplex PCR have focused on H5N1 identification in the last decade. Wu *et al.* used multiplex RRT-PCR to simultaneously detect influenza virus types A and B and for identification of subtypes H5 and N1 in a single tube reaction with four sets of primers [39]. Chaharaein *et al.* and others detected H5, H7 and H9 subtypes of AIVs by subtype-specific multiplex RT-PCR [40]. In a study of low pathogenicity avian influenza subtypes, a SYBR Green-based multiplex RRT-PCR assay was developed by Ong *et al.* for simultaneous detection and subtyping of NP, H9 and N2 genes of influenza A virus based on T_m discriminations [41]. The only US FDA approved commercial detection kit for influenza A virus, xTAG RVP Assay (Luminex Molecular Diagnostics, Inc., Toronto, Canada), is also a multiplexed RT-PCR molecular test, which is capable of detecting 20 viruses and subtypes simultaneously in a single patient sample [42].

Nucleic acid sequence-based methods

DNA microarray

DNA microarray technology, containing immobilized oligonucleotides, probes or robotically spotted DNAs, can be used to screen thousands of different nucleic acid sequences simultaneously [43,44]. It also provides a means for identifying and subtyping influenza A virus during surveillance [45,46]. A DNA array, MChip (InDevR, CO, USA), was developed for subtyping influenza A virus by targeting only the matrix gene segment of influenza A [47]. This method was then evaluated with 16 historic subtype H1N1 influenza A viruses, including A/Brevig Mission/1/1918. MChip successfully detected the matrix gene segments from all 16 viruses [48]. Townsend *et al.* developed a low-density oligonucleotide microarray (FluChip-55 [InDevR]) for the subtyping of H1N1, H3N2 and H5N1 [46]. In 2009, a new version of the respiratory pathogen microarray (TessArray RPM-Flu 3.0 and 3.1 [TessArae,

LLC, VA, USA]) was designed for the detection and differential identification of all subtypes of influenza virus HA and NA genes in a single-pass assay [49]. Huang *et al.* developed a novel assay by combining a two-step reverse transcription-multiplex PCR with typing and subtyping on a microarray, targeting the H1, H3, H5, N1, N2 and matrix genes of influenza A virus and NS gene of influenza B virus [50]. The assay can identify influenza A and B viruses and subtype influenza A virus H1N1 and H3N2, as well as the avian virus subtype A/H5N1 [49].

Pyrosequencing

Pyrosequencing is a DNA sequencing technique based on the release of pyrophosphate on incorporation of a nucleotide into a nascent DNA sequence to provide direct sequencing data. In an enzymatic reaction cascade, emitted light is generated at a level proportional to the number of incorporated nucleotides [51]. Pyrosequencing has limitations for genomic sequencing efforts due to the short average reading length, but it has been used for genotyping, resequencing disease genes and sequencing of DNA with secondary structures. As part of increased surveillance for the emergence of drug-resistant viruses, the pyrosequencing method has proven to be a powerful technique [52]. It has been used for rapidly detecting molecular markers of resistance to M2 ion-channel blockers and NA inhibitors in influenza A H5N1 viruses [53] and was also recently employed to detect and quantitate influenza NA inhibitor resistance mutations [54]. Pyrosequencing, combined with RT-PCR techniques, has provided rapid, high-throughput and cost-effective screening of NA inhibitor-resistant influenza A viruses of subtype H1N1, H3N2 and H5N1 [55]. Due to the increasing diversity among H5N1 viruses, pyrosequencing RT-PCR is also a powerful technique for distinguishing viruses belonging to the different H5 HA clades by amplifying the specifically variable regions of the HA gene [56].

Nucleic acid sequencing-based amplification method

Nucleic acid sequencing-based amplification (NASBA) is an isothermal nucleic acid amplification method that developed from transcription-based amplification systems [57]. In 2004, Lau *et al.* used this method to detect a portion of the HA gene of avian influenza A virus subtypes H5 and H7 irrespective of lineage and also used the matrix gene to detect all the then known subtypes (H1–H15) of AIV [58]. NASBA methods have also been used for surveillance of low pathogenicity and HPAI H5 AIVs [59]. In a recent study, a real-time NASBA was used for molecular detection of influenza A H1N1 and H3N2, influenza B, respiratory syncytial virus and human metapneumovirus using human respiratory samples collected on dry cotton swabs [60]. The simplicity of this method demonstrated a suitable and robust alternative technique for field influenza A virus surveillance.

Loop-mediated isothermal amplification

Loop-mediated isothermal amplification (LAMP) is a specific, efficient and rapid technique that is similar to PCR amplification but the target DNA amplification is under isothermal conditions. This method employs a DNA polymerase and a set of four specially designed primers that recognize a total of six distinct sequences on the target DNA [61]. Many experimental data have demonstrated that LAMP is a sensitive and rapid method for detection of HPAI H5N1 and other influenza viruses [62,63]. RT-LAMP was used to develop a rapid and sensitive diagnostic assay for the H9 subtype of AIV, with 10-fold higher sensitivity than that of RT-PCR, and provided a new possibility for high-throughput screening of influenza virus in wild and domestic birds [61]. It has also been used to detect and subtype influenza A virus H1 and H3 subtype strains and influenza B virus strains [64], and for HPAI H5N1 detection. The system successfully detected H5 HA genes in throat swabs collected from humans as well as from wild birds [65].

Molecular detection & surveillance for 2009 pandemic influenza H1N1 virus

On 25 April 2009, the WHO declared the outbreak of a novel influenza A virus (H1N1) as a 'Public Health Event of International Concern' under the International Health Regulations (2005) [202]. As of 11 June 2009, the WHO raised the level of pandemic alert to phase 6 after global spread of the new virus. With our limited knowledge on the virulence and evolving features of this novel H1N1 virus, early detection and genetic characterization are critical for heightened surveillance, policy decisions, vaccine drug treatment and hospital management.

In the USA, the National Respiratory and Enteric Virus Surveillance System laboratories participate in the influenza surveillance system. At the beginning of the 2009 outbreak, the serological assays most commonly used for influenza A virus detection were unable to detect the newly emerged swine-origin influenza A H1N1 virus. The CDC developed a RRT-PCR subtyping assay that targeted the novel H1N1 virus HA and matrix genes. This RRT-PCR Swine Flu Panel assay was then approved by the FDA for clinical influenza test applications and was rapidly distributed to research and clinical laboratories worldwide to ensure that information on novel influenza A H1N1 virus infection was released in a timely and accurate manner in the international community. The sensitivity of this real-time PCR to detect viral antigens in respiratory clinical specimens was evaluated and compared with RIDTs [66]. The results showed that the sensitivity of RIDTs compared to the RRT-PCR is 60–80% for seasonal A (H1N1), 80–83% for seasonal A (H3N2) and 40–69% for novel influenza A (H1N1) [65].

In addition, other PCR-based molecular protocols were also rapidly developed and used in research and clinical laboratories during the outbreak [67]. When the large outbreak of novel influenza A H1N1 virus occurred in Milwaukee (WI, USA) in late April 2009, He *et al.* developed a rapid and accurate multiplex RT-PCR-enzyme hybridization assay (FluPlex [Medical College of Wisconsin, WI, USA]) to confirm the first infected patients in the state. This assay was designed specifically for differentiating novel influenza A viruses H1N1, and for typing influenza A and B virus, as well as subtyping H1, H2, H3, H5, H7, H9, N1 (human and animal), N2 or N7 of influenza viruses [68]. As a semiautomated system, FluPlex was demonstrated to be a useful platform for high-throughput detection of influenza virus. For the purpose of clinical surveillance and control, Wang *et al.* developed a real-time protocol that can detect and distinguish both seasonal human H1N1 virus and the 2009 pandemic H1N1 virus in a single tube reaction [69]. In Hong Kong, SYBR Green I real-time PCR was employed to confirm the first human infection with 2009 pandemic H1N1 virus [70]. The 83-bp fragment of the HA gene of the virus was amplified from patient samples following a positive result with an RT-PCR test of influenza A virus matrix gene, and negative result of RT-PCR detection for influenza A virus subtypes H1 (seasonal) and H3 [69].

In response to the 2009 H1N1 pandemic, most countries have enhanced and strengthened their virological surveillance systems. PCR-based molecular methodology is employed by many health officials for tracking, detecting and characterizing H1N1 virus infection [71–73]. As part of the enhanced surveillance from the Chicago Department of Public Health, hospital laboratories in Chicago are now initiating RRT-PCR testing to characterize influenza strains [74]. National Influenza Reference Centers in France developed a H1N1 virus RT-PCR protocol to identify and investigate clusters of influenza-like illness [75]. Belgium, New Zealand, Greece and many other countries have also adopted RRT-PCR techniques for typing and subtyping influenza A, B, seasonal A (H1N1 and H3N2) and 2009 pandemic influenza A (H1N1) [76–78]. Recently, the CDC RRT-PCR assay and another three RRT-PCR assays were compared and evaluated for their specificity and sensitivity for detecting pandemic influenza A (H1N1) virus [79]. It is critical that the molecular laboratory capacity for distinguishing 2009 pandemic influenza A (H1N1) virus from other circulating influenza A viruses be expanded globally.

Highly pathogenic H5N1 surveillance

The recent lineages of highly pathogenic H5N1 viruses have been recognized in Asia since 1996 [80] when the first Asian-lineage H5N1 virus (Goose/GD/96-like) was isolated from sick geese in southern China. Since 2003, multiple reassortment events have occurred and different antigenic and genotypic clades have spread widely in domestic waterfowl and poultry in numerous countries in Asia, Europe and Africa [69,81]. H5N1 has also been isolated from wild birds in these regions [82]. So far, 492 documented human HPAI H5N1 cases have been reported with 291 (59.2%) deaths in 60 countries [203]. While chains of human-to-human transmission have not occurred with this virus, the rapid geographic expansion of H5N1, the high case–fatality rate in humans and the near 100% mortality in poultry has greatly promoted an intensified biological surveillance system in poultry and wild bird populations all over the world [83–85]. While virus isolation and serological tests are still the established gold standards, the majority of surveillance studies have been based on molecular methods such as real-time PCR, multiplex PCR and DNA array owing to their sensitivity and suitability for high-throughput analyses.

In 2006, the US Department of Agriculture, US Department of Interior, and cooperating state fish and wildlife agencies began surveillance for HPAI H5N1 virus in wild birds in the Pacific Flyway of the USA [86]. Using H5 real-time PCR, approximately 20,000 samples were screened in this study and 32 H5-positive samples were identified as H5N2, H5N3 and H5N9 but no HPAI H5N1 viruses were found. In 2004, AIV surveillance became part of the European wild bird monitoring program and has since been performed by the German Federal States in a concerted action with the National Reference Laboratory for avian influenza [87]. A total of 1991 environmental samples of fresh avian fecal matter from several aquatic bird species in a coastal area of Northeast Germany were examined for the presence of AIV by using RRT-PCR targeting the matrix gene [88]. Intensive surveillance of dead wild birds for H5N1 avian influenza infection is also conducted in Hong Kong by H5 RRT-PCR [89]. Between January 2006 and October 2007, pooled cloacal and tracheal swabs from 17,692 dead wild birds from 16 different orders (including 82 genera) were tested and 33 HPAI H5N1 viruses were isolated [87]. The nationwide H5N1 surveillance program launched in 2004 by the Government of Vietnam and implemented by the National Centre for Veterinary Diagnostics, in collaboration with the Vietnam Department of Animal Health (DAH) and DAH Regional Animal Health offices, involved the collection and analysis of 20,567 samples from 38 provinces [88]. Between that year and May 2007, a total of 2111 positive specimens were detected by RT-PCR or RRT-PCR specific for H5 HA RNA [90]. By combining with serological tests, virus culture in chicken eggs and RT-PCR methods, Shieh *et al.* in Taiwan screened 9833 animals from 1974 pig farms in nine counties for avian and swine influenza virus for the prevalence of AIV HA subtypes H1, H3, H5, H7 and swine influenza subtypes H1 and H3 [91]. In a recent report, a 384-well format for high-throughput RRT-PCR testing for avian influenza was evaluated. The sensitivity and specificity between the 384-well format and the 96-well platform are comparable [92]. This study may provide an option for high-throughput screening during an outbreak when the sample numbers and viral load are high.

Surveillance for antiviral resistance

Although vaccination plays a major role in prevention of influenza virus infection, antiviral therapy is the most important therapeutic option in treating and preventing influenza virus infections [93]. There are two classes of drugs licensed for the treatment and prophylaxis of influenza in the clinic: M2 ion channel inhibitors (amantadine and rimantadine) and NA inhibitors (oseltamivir and zanamivir). Currently, virtually all circulating strains of influenza A in the USA are resistant to either of the two major classes of anti-influenza drugs [94]. Seasonal H1N1 viruses are now resistant to oseltamivir while the 2009 pandemic H1N1 and

seasonal H3N2 viruses are resistant to the adamantanes [95]. Monitoring mutations conferring the antiviral resistance in influenza is critical to public health epidemiology and pandemic preparedness activities. At present, several molecular techniques, in addition to the standard phenotypic and genotypic analyses, have been developed for antiviral resistance detection.

A histidine to tyrosine substitution in the NA active site (H275Y) has previously been documented as a genetic marker of resistance to oseltamivir among patients with either seasonal influenza A H1N1 or influenza A H5N1 virus infections [96,97]. During the 2007–2008 influenza season, a significant rise in the percentage of oseltamivir-resistant isolates with the H275Y mutation was observed among seasonal influenza A H1N1 strains in Europe, Asia and the USA [98]. In response to this development, pyrosequencing and real-time PCR have become the most applicable high-throughput methods to detect oseltamivir resistance mutations. Carr *et al.* developed a rapid assay to detect H275Y mutation in human influenza A H1N1 using one-step allelic discrimination RRT-PCR [99]. Duwe *et al.* designed an RT-PCR assay with subsequent pyrosequencing analysis for detecting NA resistance-associated mutations in the circulating human and avian influenza A virus of subtypes of H1N1, H3N2 and H5N1 [100]. In a recent publication, a pyrosequencing approach that can detect the most common NA inhibitor resistance markers, including H275Y mutation in seasonal influenza A virus, was also developed [101]. This assay can be used with virus in clinical specimens and allows the same viral RNA preparation to be used to detect mutations for both classes of anti-influenza drugs.

Amantadine- and rimantadine-resistant strains have been found in the vast majority of recent human influenza A H3N2 strains. The V27A and S31N mutations account for up to 97% of adamantane resistance [52]. Pyrosequencing, microarray and RT-PCR have all been successfully used for monitoring the occurrence and spread of amantadine-resistant influenza virus. In a recent surveillance study for the 2006–2007 and 2007–2008 influenza seasons, a pyrosequencing method was used to screen for adamantane resistance and oseltamivir resistance mutations [102]. The study found that adamantane resistance in H3N2 virus increased from 82.7% in the 2006–2007 season to 100% in the 2007–2008 influenza season, and the adamantane resistance in H1N1 viruses increased from 0% detected in the 2006–2007 season to 1.2% detected in the 2007–2008 season.

While the circulating 2009 pandemic H1N1 strains remain susceptible to oseltamivir and zanamivir, but resistant to amantadine and rimantadine, resistance to oseltamivir has been detected in nine H1N1 isolates in the USA [103]. Using pyrosequencing, the H275Y NA mutation was detected and confirmed in patients receiving oseltamivir treatment [100]. Oseltamivir-resistant 2009 pandemic H1N1 virus isolates have also been reported in Denmark, Japan and Hong Kong [104]. These sporadic cases of oseltamivir resistance highlight the need for global surveillance of antiviral resistance for pandemic H1N1 virus.

Expert commentary

At the time of this writing, many new studies on influenza virus molecular diagnosis and subtyping continue to be published. If the outbreak of HPAI H5N1 in the last decade largely enhanced the development and application of molecular techniques for influenza A virus surveillance efforts, the first pandemic of the 21st Century extensively heightened public awareness for the importance of molecular surveillance and diagnosis for current and future pandemic preparedness. During the initial outbreak of novel H1N1, molecular detection methods rapidly became the most powerful tools for detection and identification and provided accurate genetic data for policymakers on drug treatment, vaccine development, hospital management and surveillance. While isolation of viruses in culture is still critical for antigenic

analysis and characterization of influenza viruses, real-time PCR-based assays have become the major techniques used in this public health emergency.

Many methods have been developed in laboratories around the world for detection of HPAI H5N1, H1N1 and other influenza viruses in recent years, as summarized in this review, and all these studies have demonstrated their great potential to become standard screening tools for influenza virus surveillance and diagnosis. It is important, however, to point out that most of the molecular methods available are currently still in the stage of research-based detection in the laboratory and need extensive optimization, standardization and validation before their transition from research to clinical and surveillance applications.

Multiplex real-time PCR can detect multisubtypes of influenza A virus in a single tube reaction eliminating not only operational contamination but also providing time-saving detection of influenza viruses for surveillance and detection. Degenerate primers, oligos and probes designed from evolutionally conserved regions for known influenza A viruses present the best way to identify unknown subtypes of influenza A virus by PCR and array techniques. The isothermal reactions, NASBA and LAMP possess great potential for influenza A virus surveillance in developing regions and countries. With all the advantages and disadvantages of each molecular method presented in this review (Table 1), serial and/or combination detection tests may be required for different applications. At the same time, less expensive, more convenient protocols and detection kits need to be developed for remote areas and field-based surveillance. Sample preparation and control standard selection are other critical factors affecting the immediacy, sensitivity and specificity of molecular tests for influenza A viruses. Proper documentation and processing of clinical and field specimens are also essential. Nevertheless, early detection, quick response and appropriate treatment of newly emerging influenza virus variants are key factors in the prevention and control of influenza and they are also the foundation of the WHO Influenza Global Surveillance Network.

Five-year view

There has been a dramatic breakthrough in the application of new molecular methods to influenza virus surveillance and diagnosis over the past decade. However, given the constant and rapid evolution of influenza A viruses, coupled with their frequent reassortment, diagnostic assays will need to be continually updated in the future. Standardized molecular detection protocols and systems should be used worldwide for influenza A virus diagnosis and surveillance in the future. A rapid, accurate, inexpensive and portable detection kit and equipment will be needed for future field surveillance for avian influenza A viruses. Such a detection system should allow subtyping of all 16 HA and nine NA subtypes and should have the ability to distinguish HPAI H5N1 from low pathogenicity H5N1. On the other hand, TaqMan probe and SYBR Green based real-time PCR assays should be used for routine screening in most hospital infection disease centers. These assays should be designed in a high-throughput format that can amplify the specific subtypes directly from human respiratory specimens with equipment for automatic sample preparation and enzymatic reactions. Multiplex RRT-PCR is still the most rapid, accurate and time-saving technique used for influenza A virus screening, diagnosis and mutation identification. A highly sensitive degenerate PCR system and cost-saving array system may need to be developed in the future for fast and accurate identification of any novel influenza virus subtypes during large screening and surveillance. Pyrosequencing, combined with other genomic sequencing methods, will be a critical focus for antiviral drug resistance mutation screening and future subtype identification studies. It is hoped that early detection, combined with phylogenetic analyses and epidemiologic studies, can closely predict the time and direction of future outbreaks in the coming years.

Key issues

- Molecular methods provide fast, accurate and sensitive detection and diagnosis for influenza A virus surveillance.
- Reverse transcriptase (RT)-PCR is the fundamental assay for most molecular detection systems used in influenza A virus detection and surveillance.
- Real-time RT-PCR is the most sensitive detection technique at present for fast, specific and high-throughput influenza A virus detection.
- Epidemic and/or pandemic prevention and control is largely dependent on early detection and diagnosis of initial cases.
- Surveillance for antiviral drug resistance mutations is critical for control and prevention of influenza A virus infections.
- Molecular detection and subtyping of highly pathogenic/H5N1 and 2009 pandemic influenza H1N1 virus are the most critical components of influenza A virus surveillance worldwide at the present time.
- Additional molecular-based assays for rapid influenza A virus surveillance and diagnosis are needed and must be standardized and widely adopted by public health authorities in the future.

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Table 1

Comparison of the molecular detection methods for influenza A virus surveillance.

Method	Advantages	Disadvantages	Potential for surveillance
Conventional PCR/RT-PCR	Relatively sensitive and specific; allows further sequence analysis	Not easily quantitative; not ideal for high-throughput	Time-consuming for large screens
Multiplex PCR	Relatively sensitive and specific; can test multiple targets in one assay; downstream sequence analysis is available; time and cost savings by running in single tube	Multiple primers easily cause nonspecific amplification	Most common method used for surveillance detection for identifying subtypes using different segments, especially combined with real-time PCR
Degenerate PCR	Amplify multitargets with a single primer pair; can detect novel subtypes by further sequence analysis	Challenge to design optimal degenerate primers	Ideal for initial screen on unknown samples
Probe-based real-time PCR (TaqMan®)	High sensitivity and specificity; ideal for quantitative and multiplex detections; can be high-throughput	High-cost probe and special equipment required; further sequence analysis is not generally possible	Sensitive and reliable method for clinical laboratories and core facilities
SYBR Green I-based real-time PCR	Cost-saving because no labeled probe is needed; time-saving by analysis dissociation curve for subtyping; can be high-throughput	Extensive optimization needed for high specificity; special equipment needed for operation	Once specific conditions are achieved, it is an ideal method for high-throughput surveillance application
Microarrays	Sensitive, specific and large amount of targets in single assay; very high-throughput	Expensive equipment needed; downstream analysis complex	Good for surveillance in core facility or high-level laboratory
NASBA	Relatively sensitive, specific and quantitative	Requires optimization for primer selection	Does not require expensive instruments so is a good choice for field applications especially when surveillance targets are well identified
LAMP	Simple, fast and cost-saving	Requires extensive optimization of primer design to achieve high sensitivity	A regular laboratory water bath or heat block is needed for reaction so it is an ideal detection method for remote areas and developing countries
Pyrosequencing	Accurate and sensitive; can be high-throughput	Time-consuming; sequencing length limitation	Very useful for detection of molecular markers of drug resistance

Acceptable specimens in the methods listed above include but are not limited to: virus isolates; nasopharyngeal, nose, throat and anal swab; nasopharyngeal, nasal, bronchial and throat wash.

LAMP: Loop-mediated isothermal amplification; NASBA: Nucleic acid sequencing-based amplification; RT-PCR: Reverse transcriptase-PCR.