

Diagnosis of Influenza Virus: Coming to Grips With the Molecular Era

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Influenza viruses continually circulate and cause yearly epidemics, which kill 20,000 people in an average year in the United States. Occasionally and unpredictably, pandemic influenza strains sweep the world, infecting 20% to 40% of the world's population in a single year. In 1918, the worst influenza pandemic on record caused 675,000 deaths in the United States and up to 40 million deaths worldwide. Despite the prevalence of this virus, molecular assays for influenza diagnosis, surveillance, vaccine strain selection, and research have lagged behind such assays for other common viral pathogens. The extreme genetic variability of influenza viruses makes the design of useful molecular-based assays challenging, but several different approaches have been successfully used. RT-PCR is effective for the initial diagnosis and has greater sensitivity than other available rapid assays. Molecular assays also can be used to subtype influenza isolates, and sequence analysis of hemagglutinin may assist greatly in surveillance studies and vaccine strain selection. RT-PCR for influenza also can be performed from tissue biopsy specimens for both retrospective diagnosis and research.

Key words: influenza virus, pandemic, RNA, PCR, review.

Influenza A viruses continually circulate in humans, causing yearly epidemics (mainly in the winter in temperate climates), with antigenically novel strains emerging sporadically as pandemic

viruses [1]. Influenza, one of the most common causes of respiratory infection, is significant because it is associated with high morbidity and the need for hospitalization. In the elderly and other at-risk groups, influenza infection is associated with high mortality. Because effective antiviral drugs are available to treat influenza, rapid and accurate diagnostic methods are essential.

In the United States, influenza kills 20,000 people in an average year [2]. Every 2 or 3 years, influenza epidemics boost the yearly number of deaths past the average, causing 10,000 to 15,000 additional deaths. When pandemic strains emerge, as they have every 10 to 40 years for at least several centuries, 20% to 40% of the population is infected in a single year, and the number of deaths caused by influenza can be dramatically more than average. In 1957 to 1958, a pandemic caused 66,000 excess deaths in the United States [3]. In 1918, the worst pandemic in recorded history caused approximately

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546,000 excess deaths (~675,000 total deaths) in the United States [4] and killed 20 to 40 million people worldwide [5–7].

Pandemic influenza viruses have emerged three times in this century: in 1918 (Spanish influenza), 1957 (Asian influenza), and 1968 (Hong Kong influenza) [1,8]. How and when novel influenza viruses emerge as pandemic strains are not understood. Influenza A viruses constantly evolve by the mechanisms of antigenic drift and shift [8]. The importance of predicting the emergence of new circulating influenza strains for subsequent annual vaccine development cannot be underestimated [9]. Such surveillance is the cornerstone of the World Health Organization (WHO) influenza surveillance network [10].

There is no doubt that influenza deserves to be taken seriously, even in its milder forms. The continual antigenic drift of circulating influenza strains allows new variant strains to escape acquired immunity to the virus, resulting in significant morbidity and mortality. During the last 30 years, the H3N2 subtype of influenza A introduced by the last pandemic in 1968 has caused as much excess mortality as the 1918 influenza [3]. However, it is the specter of pandemic influenza, especially pandemic influenza on the scale of 1918, that compels interest in the disease [11]. It is very likely that influenza will return in pandemic form [12]. It recently was estimated that the next influenza pandemic may result in 89,000 to 207,000 deaths, 314,000 to 734,000 hospitalizations, 18 to 42 million outpatient visits, and 20 to 47 million additional illnesses for the United States alone [13]. The estimated economic impact would be US \$71.3 to \$166.5 billion, excluding disruptions to commerce and society.

Clinical Course of Disease

Influenza is an acute viral respiratory disease characterized by the sudden onset of high fever, coryza, cough, headache, prostration, malaise, and inflammation of the upper and lower respiratory tree. Acute symptoms and fever often persist for 7 to 10 days. Weakness and fatigue may linger for weeks. It usually occurs as an epidemic in the winter (temperate climates). Persons of all ages are afflicted, but the prevalence is greatest in school-age children, and severity is greatest in very young,

aged, or immunocompromised individuals. Individuals with chronic pulmonary or cardiac disease also are at high risk for developing severe complications. Complications include hemorrhagic bronchitis, pneumonia (primary viral or secondary bacterial), and death (especially in elderly, newborn, and immunocompromised individuals). In severe cases, hemorrhagic bronchitis and pneumonia can develop within hours. Fulminant fatal viral pneumonia occasionally occurs; dyspnea, cyanosis, hemoptysis, pulmonary edema, and death may proceed in as little as 48 hours after the onset of symptoms in such cases. Pandemic influenza strains (caused by circulation of a new influenza A subtype) generally cause more severe disease, especially in persons at high risk [14].

Influenza A and B viruses are the most common causes of influenza-like illness (ILI), but other pathogens also cause ILI, including influenza C viruses, parainfluenza viruses, respiratory syncytial viruses, and *Mycoplasma pneumoniae*. At the peak of an epidemic, approximately one third of isolates from patients with ILI will be positive for influenza A [15]. It is spread by person-to-person contact. Influenza produces widespread sporadic respiratory illness every year. Acute epidemics occur every few years, generally nationwide in late fall or winter. A major shift in the prevailing subtype of influenza A with the development of a pandemic occurs on average every 30 years. Like influenza A, influenza B also can cause large epidemics. Conversely, influenza C virus is endemic and sporadically causes mild respiratory disease. This review concentrates primarily on influenza A viruses.

Influenza viral replication peaks approximately 48 hours after inoculation and declines slowly, with little virus shed after 6 days. Influenza virus replicates in both upper and lower respiratory tracts. Even after infectious virus can no longer be recovered, viral antigens can be detected in cells and secretions of infected individuals for several days. The diagnosis of influenza can be established by culture of the virus, demonstration of viral antigens or viral genetic material in clinical specimens, or increase in specific antibody titers in serum or respiratory secretions.

The only effective measure against influenza A is prevention of infection by either vaccination or prompt administration of antiviral drugs [16]. Vaccination is the most effective strategy for protection. Studies of healthy young adults have shown influ-

enza vaccine to be 70% to 90% effective in preventing illness. However, the vaccine normally gives protection for only 1 to 3 years because the viral strains that circulate change antigenicity over time. Annual revaccination is recommended for those at high risk [16]. Antiviral drugs can have both therapeutic and prophylactic effects, but have to be administered regularly at times of high influenza activity to prevent disease. Matrix 2 ion channel blockers (amantidine and rimantidine) are effective against influenza A viruses, but resistant viral strains develop rapidly with therapy and have been recognized in approximately one third of treated patients [17]. The more recently developed neuraminidase (NA) inhibitors (zanamivir and oseltamivir) are effective against both influenza A and B viruses. They have been approved only for persons symptomatic with influenza for less than 2 days [16]. However, both drugs have been shown to be effective in preventing influenza when administered prophylactically [18,19].

Biology of Influenza Viruses

Influenza viruses (Orthomyxoviridae) are enveloped negative-strand RNA viruses with a segmented genome. They are divided into two genera: one including influenza A and B and the other consisting of influenza C, based on significant antigenic differences between their nucleoprotein and matrix proteins. The three virus types also differ in pathogenicity and genomic organization. Type A is found in a wide range of warm-blooded animals, but types B and C are predominantly human pathogens [14].

Influenza A viruses are further subdivided by antigenic characterization of the hemagglutinin (HA) and NA surface glycoproteins that project from the surface of the virion. There are currently 15 HA and nine NA subtypes [12]. Influenza A viruses infect a wide variety of animals, including birds, swine, horses, humans, and other mammals. Aquatic birds serve as the natural reservoir for all known subtypes of influenza A and probably are the source of genetic material for human pandemic influenza strains.

Unlike the related paramyxoviruses, influenza viruses have a segmented RNA genome. Influenza A and B viruses have a similar structure, whereas influenza C is more divergent. A and B type viruses

each contain eight discrete gene segments coding for at least one protein each. Influenza A and B viruses are covered with projections of three proteins: HA, NA, and matrix 2 (M2; Fig. 1). Influenza C viruses have seven segments and only one surface glycoprotein [20].

Each influenza RNA segment is encapsidated by nucleoproteins (NP) to form ribonucleotide-nucleoprotein (RNP) complexes. The three polymerase proteins are associated with one end of the RNP complex. RNPs are surrounded by a membrane with the matrix protein (matrix 1) as an integral part. The phospholipid portion of the envelope is derived from the cellular host membrane. Also found within the virus particle is nonstructural protein 2 (NS2; Fig. 1) [20].

WHO guidelines for nomenclature of influenza viruses are as follows. First, type of virus is designated (A, B, or C), then the host (if nonhuman), place of isolation, isolation number, and year of isolation (separated by slashes). For influenza A, HA and NA subtypes are noted in parentheses. For example, strains included in the most recent trivalent vaccine for the 2000 to 2001 season are: A/Panama/2007/99 (H3N2), A/New Caledonia/20/99 (H1N1), and B/Yamanashi/16/98 [15]. Since 1977, there have been two influenza A subtypes cocirculating in humans: H1N1 and H3N2 (Table 1).

Influenza viruses accumulate point mutations during replication because their RNA polymerase complex has no proofreading activity. Mutations that change amino acids in the antigenic portions of surface glycoproteins may give selective advantages for a viral strain by allowing it to evade preexisting immunity. The HA molecule initiates infection by binding to receptors on certain host cells. Antibodies against the HA protein prevent receptor binding and are very effective at preventing reinfection with the same strain.

HA can evade previously acquired immunity by either antigenic drift, in which mutations of the currently circulating HA gene disrupt antibody binding, or antigenic shift, in which the virus acquires HA of a new subtype [14]. Antigenic drift pressures are unequal across the HA molecule, with positively selected changes occurring predominantly on the globular head of the HA protein. These changes also accumulate to a greater extent in HA than NA. Changes in other influenza proteins occur more slowly. Likewise, antigenic drift pressure is greatest in human-adapted influenza strains,

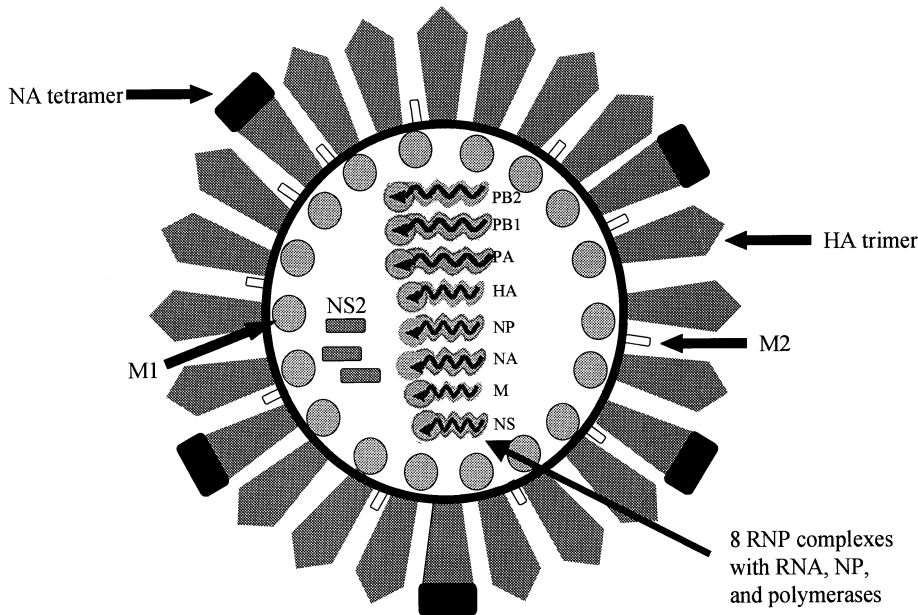


Fig. 1. Diagrammatic representation of an influenza A virus. The two major surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA), are embedded in a lipid bilayer. Small numbers of the matrix 2 (M2) ion channel protein are inserted into the lipid envelope. The matrix 1 (M1) protein underlies the envelope and interacts with helical ribonucleoproteins (RNPs). RNPs consist of the eight negative-stranded RNA segments and nucleoprotein (NP) and small amounts of the polymerase complex (PB2, PB1, and PA) [20]. NS2, nonstructural protein 2.

intermediate in swine- and equine-adapted strains, and least in avian-adapted strains [14].

Because influenza viruses have a segmented genome, coinfection with two different strains in the same host can lead to the production of novel reassorted influenza strains containing different combinations of parental gene segments. Fifteen HA subtypes are known to exist in wild birds and provide a source of HAs that are novel to humans [12]. The emergence in human circulation of an influenza strain with a novel subtype by antigenic shift has been the cause of the last two influenza pandemics in 1957 and 1968 and was most likely the cause of the 1918 influenza pandemic (Table 1).

To be concordant with all that is known about the emergence of pandemic influenza viruses, a pandemic strain must have an HA antigenically distinct

from the one currently prevailing; this HA cannot have circulated in humans for 60 to 70 years; and the virus must be transmissible from human to human [21]. In both 1957 and 1968, pandemics resulted from a shift in HA, and in both cases, HAs of pandemic strains were closely related to avian strains. Although one of the absolute requirements for a pandemic is that HA must change, the extent to which the rest of the virus can or must change is not known. Only the pandemic viruses of 1957 and 1968 are available for direct study. (The 1918 pandemic influenza virus is being characterized using molecular archeology, discussed later). In 1957, three genes were replaced by avian-like genes: HA, NA, and a subunit of the polymerase complex (PB1). In 1968, only HA and PB1 were replaced [22,23].

Table 1. Influenza A Virus Circulation in Humans

Year	Outbreak	Subtype	Period of Circulation
1889-1892	Pandemic	H3N? (archeserology)	1889-1918? [82]
1918-1919	Pandemic	H1N1	1918-1957
1957-1958	Pandemic	H2N2	1957-1968
1968-1969	Pandemic	H3N2	1968-present
1976	Limited outbreak*	H1N1 (swine)	1976
1977	Epidemic	H1N1 (re-emergence of pre-1957 H1N1 strain)	1977-present, cocirculation with H3N2
1997	Limited outbreak*	H5N1 (avian)	1997

*Swine H1N1 isolated from a small number of soldiers in Fort Dix, NJ, in 1976 causing a small outbreak with one death. In 1997, a chicken H5N1 influenza strain was isolated from 18 people in Hong Kong, with six deaths.

Although an antigenically novel HA subtype is a requirement for the emergence of an influenza pandemic, human infection with an animal-adapted influenza virus of novel subtype alone is not sufficient for the development of pandemic strain. There are two cases in point. In the exposure of a limited number of soldiers to a wholly swine H1N1 influenza virus in Ft Dix, NJ, in 1976, there was one death, and the concern that this strain may cause a pandemic led to the swine flu vaccination program in 1976 to 1977 [24,25]. Similarly, 18 people were exposed to a chicken-adapted H5N1 virus in Hong Kong in 1997, and six people died of complications of infection [26,27]. Both these examples show that although humans can be infected zoonotically with animal-adapted influenza viruses without reassortment and infection may lead to fatal complications, the viruses seemingly spread too inefficiently from person to person to cause a pandemic directly. However, the risk of these outbreaks is the possible recombination through antigenic shift of the zoonotically transferred influenza strain with the circulating human-adapted strain to produce a pandemic strain containing human-adapted influenza core proteins and novel surface proteins.

Classic Diagnostic Techniques

A specific diagnosis of influenza infection can be made by virus isolation, hemagglutination inhibition (HI) test, antigen detection by immunoassay, serological tests, demonstration of NA activity in secretions, or molecular-based assays. Specimens can be collected as sputum, nasopharyngeal swab, or nasopharyngeal washing obtained by gargling a buffered-saline solution. The standard for influenza diagnosis has been immunologic characterization after culture [28].

Serological analysis provides an accurate but retrospective method for influenza infection because it requires collection of both acute and convalescent sera. Thus, although this method is very useful epidemiologically for strain surveillance, it cannot be used for rapid diagnosis that would allow therapeutic intervention in a patient or timely prophylaxis of at-risk contacts [29].

Influenza viruses can be grown in embryonated hens' eggs or a number of tissue culture systems. The addition of trypsin (for the cleavage activation of HA) allows influenza virus propagation in Madin-

Darby canine kidney (MDCK) cells and other lines. The primary method for vaccine production is still the cultivation of influenza viruses in eggs. Culture in cell lines is commonly used for the primary isolation of human influenza viruses (both types A and B). Many human influenza viruses can be cultivated directly in the allantoic cavity of embryonated eggs. Some influenza A and B viruses require initial cultivation in the amniotic cavity and subsequent adaptation to the allantoic cavity. After culture isolation, most influenza isolates are definitively identified using immunoassays or immunofluorescence [14].

HA molecules of influenza viruses bind sialic acid residues on the surface of respiratory cells for the virus to gain entry [8]. Influenza strains can be characterized antigenically by taking advantage of the ability of influenza viruses to agglutinate erythrocytes *in vitro*. Anti-HA antibodies can inhibit agglutination [28,29]. Thus, HI assay is one of the standard methods used to characterize influenza strains. Because the method is labor intensive, many laboratories postpone subtyping analysis until after the influenza season, which may considerably delay accumulation of relevant epidemiological data [30]. HI assays are used to determine whether sample strains are immunologically related (i.e., cross-reactive) to recent vaccine strains. Typing sera, generally produced in ferrets, are added to wells in a series of two-fold dilutions, and laboratory workers score assay wells (\pm) by looking for suspended versus clumped red blood cells. In most situations, a panel of sera is used for matching sample strains against vaccine and reference strains, and during any given influenza season, the vast majority of sample strains are successfully matched by HI assays [15]. Sample strains are categorized according to immunologic pedigrees, such as A/Moscow/10/99 (H3N2)-like, A/New Caledonia/20/99 (H1N1)-like, and B/Beijing/184/93-like viruses. For sample strains that fail characterization in HI assays, laboratory workers must inoculate them into ferrets to produce a strain-specific antiserum. When the new antiserum is ready, HI assays are performed again as described. If the new serum shows significant gaps in cross-reactivity (usually defined as a fourfold difference between sample and vaccine strains), it is incorporated into the routine laboratory panel and used to look for new epidemic strains. Thus, HI assays are extremely important in the influenza virus surveillance effort for vaccine strain selection and are the most commonly used methods to assess antigenic drift [30].

Although influenza virus culture has been the standard for influenza diagnosis by which other techniques are measured, it often takes 5 to 10 days to perform. To make effective use of antiviral therapies, more rapid diagnostic strategies are needed. Such techniques include immunofluorescence-based assays (IFAs) and other immunoassays (e.g., enzyme immunoassay [EIA], radioimmunoassay, enzyme-linked immunosorbent assay) that can be performed directly on clinical material (generally nasopharyngeal swabs or aspirates) to detect specific antigen. Such tests have sensitivities in published trials of 60% to 90% and specificities greater than 90% compared with viral culture [31–36]. Of these tests, IFA generally has very high sensitivity, but is labor intensive and requires expertise in fluorescence microscopy [37]. Four commercially available rapid diagnosis test kits are marketed for influenza, with sensitivities compared with culture isolation of approximately 75%. One test detects NA activity in clinical specimens [36], whereas the other three are immunoassays. Three tests do not distinguish between influenza A and B, and the fourth test can distinguish influenza A and B. Three of these tests have the advantage of point-of-care testing, allowing a real-time rapid diagnosis to be made in a clinical setting (Table 2).

Molecular Diagnostic Assays

RT-PCR for Influenza Diagnosis

Because of limitations of current methods, laboratory confirmation of influenza infection is performed infrequently in the clinical setting. However, the availability of effective (if administered early) antiviral drugs requires the development of rapid and sensitive diagnostic techniques. Furthermore, although the characterization of influenza strains by public health laboratories as part of the global WHO surveillance network for influenza vaccine strain selection is the most comprehensive infectious disease surveillance program in the world, it covers only a minute fraction of suspected influenza cases annually. Last year, approximately two thirds of influenza-positive samples collected were typed as A or B, but only half the influenza A isolates were subtyped. Thus, rapid, sensitive, and inexpensive assays for influenza diagnosis and characterization are needed for both rapid patient diagnosis and global surveillance.

RT-PCR amplification of influenza virus RNA as a diagnostic assay was first reported in 1991 [38]. In the next decade, additional diagnostic RT-PCR assays for influenza types A, B, and C have been de-

Table 2. Laboratory Diagnostic Procedures for Influenza

Procedure	Influenza Types Detected	Acceptable Specimens	Time for Results	Point-of-care Test
Viral culture	A and B	NP swab, throat swab, nasal wash, bronchial wash, nasal aspirate, sputum	5-10 d	No
Immunofluorescence	A and B	NP swab, nasal wash, bronchial wash, nasal aspirate, sputum	2-4 h	No
Influenza A enzyme immunoassay	A and B	NP swab, throat swab, nasal wash, bronchial wash	2 h	No
Directigen Flu A+B (BD Diagnostic Systems, Sparks, MD)	A and B	NP swab, throat swab, nasal wash, bronchial wash	2 h	No
FLU OIA (Thermo Biostar, Boulder, CO)	A and B (does not distinguish)	NP swab, throat swab, nasal aspirate, sputum	30 min	Yes
Quick Vue (Quidel, San Diego, CA)	A and B (does not distinguish)	NP swab, nasal wash, nasal aspirate	30 min	Yes
Zstat Flu (ZymeTx, Oklahoma City, OK)	A and B (does not distinguish)	Throat swab	30 min	Yes
RT-PCR	A and B (and C)	NP swab, throat swab, nasal wash, bronchial wash, nasal aspirate, sputum	1-2 d	No
Serology	A and B	Paired acute and convalescent serum samples*	2-4 wk	No

NP, nasopharyngeal.

*Fourfold or greater increase in antibody titer from the acute sample (collected within first week of illness) to the convalescent-phase sample (collected 2 to 4 weeks after the acute sample) is indicative of recent infection.

scribed [39–41], with numerous reports published in the last year [42–47]. Because of their sensitivity and quick turnaround time compared with traditional diagnostic assays, RT-PCR–based assays could have a significant role in influenza diagnosis if they can be transferred out of the realm of research laboratories and into clinical laboratories.

In five recent studies comparing RT-PCR for influenza A and B with viral isolation, 1,298 samples (consisting of nasopharyngeal swabs or aspirates) from patients with ILI were collected. Of these, 550 samples (42.4%) were positive by RT-PCR for either influenza A or B compared with 401 samples positive (30.9%) by viral culture [40,43,44,46,47]. Four of these studies used primers published in 1991 [38] for typing samples as influenza A or B using conserved matrix gene primers and primers designed to the HA and NA genes for subtyping of influenza A specimens (H3N2 or H1N1) [40,43,44,46]. One study used almost identical primer sets from H3 and H1 subtype genes of influenza A and the HA gene of influenza B [47]. In all cases, nested RT-PCR strategies were used.

Quantitative Real-time PCR for Influenza Diagnosis

In the previously mentioned studies using nearly identical nested primer sets, the sensitivity of RT-PCR for influenza diagnosis was shown. However, concern about contamination in nested PCR assays for clinical use recently led other groups to develop such real-time fluorogenic PCR-based strategies as TaqMan (Applied Biosystems, Foster City, CA) for influenza diagnosis [48,49]. In these assays, oligonucleotide probes internal to the PCR primers are labeled with fluorescent reporter and quencher dyes. On probe hybridization, the 5'-3' exonuclease activity of Taq polymerase cleaves the fluorescent probe away from its quencher, allowing fluorescence to be quantitated in the reaction in real time. This technique also has the advantage of reducing PCR contamination because confirmatory probe hybridization occurs during amplification in a closed tube and does not require postamplification analysis. Another advantage is the rapidity of the assay; results can be available in just a few hours after obtaining a clinical sample.

Schweiger et al. [48] collected 2,545 samples from patients with ILI over a 2-year period in

Germany. Using their TaqMan assay, which can distinguish influenza A from influenza B, they obtained 720 positive samples (28%) compared with 406 positive samples (15%) obtained by viral isolation. Samples collected in the community for epidemiological and surveillance studies often arrive in the laboratory several days after collection. This may partly explain the lower yield of culture-isolated influenza viruses on this study. Influenza typing was performed using conserved primers and probes from the matrix 1 gene of influenza A and the HA gene of influenza B. Influenza A samples then were subtyped using HA and NA primer/probe sets for H1N1 and H3N2 viruses. The sensitivity of the assay (all primer/probe sets) was approximately 0.1 of the 50% tissue-culture infectious dose, representing approximately 10 virions/reaction. Specificity was confirmed using conventional typing and subtyping methods and the type- and subtype-specific nested RT-PCR assays described previously.

PCR Primer Selection in the Face of Influenza Sequence Variation Over Time

Because influenza viruses continually evolve by antigenic drift and random mutation, this must be considered in the design of PCR-based assays, especially those for the HA gene. The RT-PCR and TaqMan PCR assays described used primers in relatively conserved portions of HA. Even so, significant variation in these sequences can be observed. An example of sequence variation in the region of the primer used as the reverse primer for the TaqMan assay described by Schweiger et al. [48] is shown in Fig. 2. Laboratories performing RT-PCR for influenza should examine recent influenza sequences and adjust primer sequences accordingly.

DNA Array for Influenza Diagnosis

Another approach to molecular-based influenza diagnosis is to develop a DNA microarray containing probes corresponding to influenza genes. Such array-based hybridization systems could theoretically identify not only influenza A and B viruses, but other infectious agents in the differential diagnosis of ILI, such as respiratory syncytial virus, parainfluenza viruses, and adenoviruses. They also

Primer HA3-375	CTG	GAG	TTT	ATC	AAT	GAA	GGC	TTC
A/Hongkong/1/68	---	---	---	---	-C-	--G	-T-	---
A/Queensland/7/70	---	---	---	---	-C-	--G	-T-	---
A/Memphis/102/72	T--	---	---	---	---	---	---	---
A/Victoria/3/75	---	---	---	---	---	---	---	---
A/Philippines/2/82	T--	---	---	---	---	---	---	---
A/Yamanashi/797/85	---	---	---	---	---	---	-A-	---
A/Shanghai/11/87	---	---	---	---	---	---	-A-	---
A/Washington/15/91	---	---	---	---	---	---	-A-	---
A/Shiga/2/91	T--	---	---	---	---	---	-A-	---
A/Texas/17988/84	---	--A	---	-C-	---	---	---	---
A/Brazil/43/97	---	--A	---	-C-	---	---	---	---
A/Florida/4/95	---	---	---	-C-	---	---	---	---
A/Wuhan/359/95	---	---	---	-C-	---	---	---	---
A/Sydney/5/97	---	---	---	-A-	---	---	A--	---
A/Taiwan/45/98	---	---	---	-A-	---	---	A--	---
A/Panama/2007/99	---	---	---	-A-	---	---	A--	---
A/Stockholm/2/00	---	---	---	-A-	---	---	A--	---

Fig 2. Variation in influenza HA sequence at a PCR primer site. Primer HA3-375 is the reverse primer for the H3 subtype TaqMan-based assay described by Schweiger et al. [48]. Representative strains obtained from GenBank are aligned with the primer sequence (presented in the sense orientation).

could subtype all 15 HA subtypes and all 9 NA subtypes of influenza A. Such arrays could be used not only for rapid and accurate diagnosis for individual patients, but in large-scale surveillance studies and to identify zoonotic exposure to animal influenza viruses (e.g., the 1997 H5N1 chicken influenza virus in Hong Kong). Unfortunately, such universal ILI arrays have not yet been developed.

However, a first step was taken earlier this year with a publication describing an influenza array containing probes for typing influenza A and B and subtyping H1N1, H2N2, and H3N2 influenza A viruses [50]. The array produced on this study contained 24 probes derived from cloned PCR-amplified fragments of influenza A matrix, HA (H1, H2, and H3), and NA (N1 and N2) genes and of influenza B matrix, HA, and NA genes. The probes averaged 500 bp (range, 154 to 810 bp). Target influenza complementary DNA was fluorescently labeled with Cy3- or Cy5-dCTP during amplification. Amplified product was hybridized to the array, washed, and analyzed using confocal scanners and commercial software. The investigators first showed that fluorescently labeled probes derived from the original cloned templates were able to hybridize to the cognate spots. Little cross-hybridization to unrelated spots was detected. Subsequently, they were able to show that complementary DNA from three test strains, including an H1N1, H3N2, and influenza B strain, were able to hybridize to the correct probes on the array. Clearly, more extensive studies are needed, but the tech-

nique may prove fruitful for influenza diagnosis and surveillance.

Restriction Fragment Length Polymorphism Analysis of Influenza Viruses

Influenza strains used for vaccine production are often reassorted *in vitro* with master strains to yield high-growth strains containing the HA gene of the desired strain [51]. To screen recombinant influenza viruses rapidly for successful gene-segment combinations of the master donor strain and current epidemic strain, post-PCR restriction fragment length polymorphism assays have been used [51,52]. More recently, a technique for molecular genotyping of the internal gene segments of H1N1, H3N2, and H5N1 influenza viruses was described [53]. Genotyping was based on subtype-specific restriction fragment length polymorphism patterns within the amplified portions of each gene segment.

Sequence Analysis of HA for Analysis of Antigenic Drift and Vaccine Strain Selection

As a correlate to antigenic analysis by HI titer, the HA1 domain of HA also was sequenced from clinical influenza A isolates [54–56]. Sequence analysis has shown that egg-adaptation mutations occur in the HA gene of human influenza strains grown in eggs compared with their growth on mammalian MDCK cells [56,57]. Because of the development of culture-dependent sequence mutations, vaccine strain selection might benefit from sequence analysis of HA performed directly from clinical samples. Recently, phylogenetic analyses of large numbers of HA1 domain sequences from H3N2 viruses have been undertaken to predict the evolution of influenza A [58–60].

RT-PCR for Influenza Diagnosis in Animals

The threat of a new influenza pandemic as a result of zoonotic infection of humans with animal-derived influenza viruses has prompted investigators to design molecular screening techniques that would identify a variety of influenza A viruses. Diagnostic screening methods for influenza virus (IFA, immunoassay, RT-PCR) were designed specifically to

identify human influenza virus strains and thus may be unable to detect avian, equine, or swine strains. Fouchier et al. [45] described an RT-PCR method using primers designed from a conserved portion of the matrix gene followed by dot-blot hybridization that was able to detect a genetically diverse set of influenza strains isolated from birds, horses, pigs, seals, and humans. RT-PCR assays designed specifically to diagnose influenza virus infection in swine [61], horses [62], and birds [63] have been described in the veterinary literature.

Utility of Molecular Assays

Molecular genetic assays have shown great utility in influenza diagnosis in a number of settings. In the future, RT-PCR and TaqMan assays may contribute greatly to the rapid and specific diagnosis of influenza infection, allowing for therapeutic intervention and prophylactic treatment of high-risk contacts. A good example of this is the rapid diagnosis of influenza in a long-term geriatric care facility, followed by prompt prophylactic antiviral treatment of the resident population.

Molecular diagnosis also will have a large role in epidemiologic surveillance, vaccine strain selection, and surveillance for the emergence of novel influenza viruses in humans, such as the H5N1 outbreak in Hong Kong. Finally, molecular genetic analysis will provide researchers with the ability to relate genotypic information to host range [64], virulence [65,66], or other clinical factors of influenza infection.

Molecular Genetic Analysis of the 1918 Spanish Influenza Virus

An example of molecular genetic analysis of an influenza virus sequence to understand virulence is the ongoing effort to sequence the genome of the influenza virus that caused the so-called Spanish influenza pandemic. The pandemic spread explosively in fall and winter 1918 to 1919, infecting 500 million people and killing 40 million [5,6]. The virus responsible for this catastrophe was not isolated because influenza viruses were not known to exist at the time. However, it recently became possible to study genetic features of the 1918 virus using frozen and fixed archival autopsy tissue [7,67–69]. Gene sequences of the 1918 virus can be used to frame

hypotheses about the origin of the 1918 virus and look for clues to its virulence [70,71]. Understanding the genetic makeup of the most virulent influenza strain in history may facilitate the prediction and prevention of future pandemics.

The main wave of the global pandemic occurred in September through November 1918, killing more than 10,000 people per week in some US cities. Almost one third of the US population became ill. The disease also was exceptionally severe, with mortality rates greater than 2.5% among the infected compared with less than 0.1% in other influenza epidemics. Incredibly, some isolated populations had mortality rates greater than 70% [5].

In the 1918 pandemic, most deaths occurred among young adults, a group that usually has a very low death rate from influenza. Influenza and pneumonia death rates for 15- to 34-year-olds were more than 20 times greater in 1918 than in previous years, with 99% of excess deaths among people younger than 65 years [3]. It was estimated that the influenza epidemic of 1918 killed 675,000 Americans, including 43,000 servicemen mobilized for World War I. The impact was so profound that it depressed average life expectancy in the United States by more than 12 years and may have had a significant role in ending World War I [5].

The majority of individuals who died during the pandemic died of secondary bacterial pneumonia because no antibiotic was available in 1918. However, a subset died rapidly after the onset of symptoms, often with either massive acute pulmonary hemorrhage or pulmonary edema. In the hundreds of autopsies performed in 1918, primary pathological findings were confined to the respiratory tree, and death was caused by pneumonia and respiratory failure [72–74]. These findings are consistent with infection by a well-adapted influenza virus capable of rapid replication throughout the entire respiratory tree [70].

Frozen and fixed lung tissue from three 1918 influenza victims has been used to examine directly the genetic structure of the 1918 influenza virus. Two of the cases analyzed were US Army soldiers who died in September 1918 in New York and South Carolina. A third sample was obtained from an Alaskan Inuit woman who had been interred in permafrost since November 1918. Amplification and sequencing of small overlapping RNA fragments extracted from these tissues allowed com-

plete viral gene sequences to be determined for the two surface protein-encoding genes, HA and NA. These sequences confirmed that the 1918 strain was an H1N1 subtype influenza A virus [7,68].

Neither the 1918 HA nor NA genes have obvious genetic features that can be related directly to virulence. Two known mutations that can dramatically affect the virulence of influenza strains have been described. For viral activation, HA must be cleaved into two pieces, HA1 and HA2, by a host protease. Some avian H5 and H7 subtype viruses acquire a mutation that involves the addition of one or more basic amino acids to the cleavage site, allowing HA activation by ubiquitous proteases. Infection with such a pantropic strain causes systemic disease in birds with near-uniform mortality [75]. This mutation was not observed in the 1918 virus [7,67]. The second mutation with a significant effect on virulence through pantropism has been identified in the NA gene of two mouse-adapted influenza strains. Mutations at a single codon, such as the HA cleavage site mutation, appear to allow the virus to replicate in many tissues outside the respiratory tract [7,67,76]. This mutation also was not observed in the 1918 virus [68].

Therefore, neither surface protein-encoding gene has known mutations that would allow the virus to become pantropic. Because clinical and pathological findings in 1918 showed no evidence of replication outside the respiratory system, mutations allowing the 1918 virus to replicate systemically would not be expected. However, the relationship of other structural features of these proteins (aside from their presumed antigenic novelty) to virulence remains unknown. In their overall structural and functional characteristics, the 1918 HA and NA are avian-like, but they also have mammalian-adapted characteristics.

Recently, the complete NS segment sequence coding for NS1 and NS2 proteins was completed [69]. It has been shown that influenza A virus NS1 protein functions as a type I interferon antagonist [77] and is required for influenza A virus virulence. One distinctive clinical characteristic of the 1918 influenza is the ability to produce rapid and extensive damage to the respiratory epithelium [73,74]. Such a clinical course suggests a virus that replicated to a high titer and spread quickly from cell to cell. The lack of an antibody-based immune response because of novel HA and NA proteins provides only a partial explanation of this phenom-

enon because other influenza strains with novel surface proteins, such as the 1957 pandemic, were not nearly as virulent as the 1918 strain. An NS1 protein that was especially effective at blocking the type I interferon system might have contributed to the exceptional virulence of the 1918 strain.

Genetic characteristics of NS1 that contribute to its ability to block interferon have not yet been mapped, and the likelihood that the 1918 NS1 would be effective at blocking interferon could not be assessed simply by examining its sequence. Therefore, to begin to understand the role the NS gene products NS1 and NS2 (nuclear export protein) may have had in virulence, the 1918 virus NS1 gene and entire 1918 virus NS segment were reconstructed, and transfectant influenza viruses bearing these genes were generated [69]. In the first set of viruses, the open reading frame encoding the 1918 NS1 or a control NS1 protein was introduced into virus. In the second set, the entire 1918 NS segment was introduced into virus. The virulence of these viruses in mice was determined.

In both cases, viruses containing 1918 NS genes were attenuated in mice compared with wild-type A/WSN/33 controls [69]. The attenuation shows that NS1 is critical for the virulence of A/WSN/33 in mice. The 1918 NS1 varies from that of WSN at ten amino acids. Amino-acid differences between the 1918 and A/WSN/33 NS segments may be important in the adaptation of the latter strains to mice and likely account for observed differences in virulence in this set of experiments. Thus, further experiments using different viral and animal backgrounds may be necessary to test the hypothesis that the 1918 NS1 specifically contributed to virulence.

Because virulence cannot yet be adequately explained by sequence analysis of the 1918 HA, NA, and NS genes, what can these sequences tell us about the origin of the 1918 virus? The best approach to analyzing the relationships among influenza viruses is phylogenetics, in which hypothetical family trees are constructed that use available sequence data to make assumptions about the ancestral relationships between current and historical influenza strains. Because influenza genes are encoded by eight discrete RNA segments that can move independently between strains by the process of reassortment, these evolutionary studies must be performed independently for each gene segment.

A comparison of the complete 1918 HA and NA genes with those of numerous human, swine, and

avian sequences showed that the 1918 sequences are placed near the root of the mammalian clade, suggesting that both surface protein-encoding genes emerged from an avian reservoir just before 1918 [7,68,71]. Clearly, by 1918, the virus had acquired enough mammalian-adaptive changes to function as a human pandemic virus. Identifying the minimal changes necessary to allow a virus with avian surface proteins to replicate and be transmitted efficiently in mammalian hosts is extremely important for our understanding of the emergence of pandemic influenza viruses.

Fragmentary sequences of all remaining gene segments of the 1918 virus have already been deciphered, and full-length segment sequences will be completed for several more 1918 influenza genes in the near future. Such sequences will allow complete phylogenetic analyses of each segment and help elucidate the origin of the 1918 virus. Whether particular genetic features of the virus can be related directly to its exceptional virulence is unclear. Even as the genetic structure of the Spanish influenza virus is becoming fully known, other questions, such as the role of differences in immunity in different age groups in 1918, may prove important. It is hoped that knowledge gained by studying this exceptionally lethal human pathogen can be applied to prevent, or at least predict, the emergence of new influenza viruses with pandemic potential [78].

Future Prospects: Global Surveillance Laboratory

The WHO Influenza Program was established in 1948 to deal with the public health threat of epidemic and pandemic influenza. Today, its network of 110 national centers in 83 countries collects approximately 175,000 samples each year from an estimated 600 to 1,200 million cases of influenza worldwide [10]. The centers screen samples for influenza viruses and also type (A versus B)- and subtype (A/H1N1, A/H3N2)-relevant samples. Certain influenza-positive samples are then forwarded to one of four WHO collaborating centers for further immunologic characterization. At 6-month intervals, an advisory committee identifies three circulating strains that should be targeted by new vaccine formulations [15]. Because influenza epi-

demics peak during winter months, the committee offers its recommendations in February and September for the northern and southern hemispheres, respectively. Such timing gives vaccine manufacturers on either side of the equator approximately 6 months for scale-up, production, and distribution. It also gives healthcare services another 3 months to administer the trivalent vaccine to those waiting, and in the final analysis, approximately 200 million doses of vaccine are administered every year.

However, despite its sophistication and scale, the WHO Influenza Program has several serious shortcomings. First, current laboratory methods to characterize influenza are time consuming and labor intensive, and as a result, relatively few viral strains undergo definitive phenotyping and genotyping assays. Second, with current methods, it sometimes takes weeks to months to generate laboratory data on influenza samples and understand their significance. Such prolonged times can impede vaccine strain selection activities [30].

Recently, a plan to create a new kind of high-throughput laboratory to expedite influenza surveillance and intervention efforts on a global scale has been developed [79,80]. The plan integrates available biological, engineering, and informatic technologies into a centralized facility and makes them available through the Internet [81]. The first system will screen native samples for common respiratory viruses, including influenza A and B, respiratory syncytial virus, parainfluenza, and adenovirus. Influenza-negative samples will be set aside, but results of such tests will be stored in the database. The second system will use replicated influenza stocks from screened viruses to characterize physical and chemical properties of influenza A and B samples. The third system will use replicated influenza stocks and native strains to perform sequencing procedures [80].

Development of such a global laboratory would greatly enhance the current influenza surveillance system, allowing for the characterization of much larger numbers of influenza strains. Such an approach is likely to identify antigenic drift variants earlier and provide for more timely vaccine strain selection. Second, the system would more readily identify unusual influenza virus exposures, such as the zoonotically transmitted H5N1 viruses, which pose a continual threat of developing into strains with pandemic potential. Finally, sequence analysis of large numbers of strains may allow the detection

of genotypic-to-phenotypic correlations that are currently impossible because of the small number of sequences available and lack of associated clinical and epidemiological data.

Conclusions

The development of rapid, sensitive, and inexpensive techniques for influenza diagnosis will have a great impact in several areas: (1) rapid diagnosis in patients with ILI for the administration of appropriate and timely therapy, (2) molecular assays to facilitate characterization of influenza strains for vaccine strain selection, and (3) molecular assays to improve the global influenza surveillance system.

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