

Laboratory surveillance of co-circulating respiratory viruses and enteroviruses during the 2009 H1N1 influenza pandemic

J. Tan, and M.P. Carlos, DVM, PhD.

Maryland Department of Health and Mental Hygiene, Laboratories Administration, Baltimore, MD 21201, USA

During the 2009 influenza A (H1N1) pandemic, many people presented at emergency rooms and clinics with influenza-like illnesses (ILI) but many of them were infected by non-influenza respiratory viruses. Enhanced surveillance and diagnosis of etiological agents of ILI has the potential to guide timing of public health interventions and to reduce healthcare costs. Our laboratory routinely tests specimens submitted by more than 100 sentinel sites of the Maryland influenza outpatient surveillance network (ILINet) for respiratory viral pathogens that can cause ILI symptoms. Molecular testing is used for laboratory identification of influenza virus while detection of other respiratory viruses is performed using culture-based method. From May 2009–April 2010, 8370 specimens were submitted for respiratory virus testing. 4998 (60%) were negative for influenza viruses. Of the 3372 positives, 3257 (97%) were 2009 pandemic influenza A (H1N1); and 56 (2%) were seasonal influenza A or B viruses. Selected PCR negative specimens were inoculated in cell culture for identification and subsequently tested by immuno-fluorescent assay for the presence of non-influenza respiratory viruses. 164 viruses including parainfluenza viruses, respiratory syncytial viruses, adenoviruses, rhinoviruses and enteroviruses were found to be the etiological agents. Expanded testing capacity in the MD DHMH to support pandemic influenza surveillance since late April 2009 greatly increased our ability to conduct laboratory testing. Active surveillance of a wide range of respiratory viral pathogens allows the continual monitoring of circulating viral strains in Maryland.

Keywords pandemic influenza; respiratory virus; influenza-like illness (ILI)

1. Introduction

Influenza infection is a common cause for acute respiratory infection; however, infections by many other pathogens may manifest influenza-like illnesses (ILI) symptoms. Studies have shown that influenza-like illnesses are actually caused by the influenza virus in only 20 percent to 70 percent of cases during the influenza season [1]. Therefore, laboratory identification of influenza and other respiratory viruses will facilitate prompt detection and characterization of etiologic pathogens and accelerate the implementation of effective public health responses. Moreover, the identification of circulating respiratory agents in long term and assisted care centers, schools or daycares provides useful surveillance data for viral epidemiology in the community [2].

In the United States, about 80 U.S. WHO collaborating laboratories and 70 National Respiratory and Enteric Virus Surveillance System (NREVSS) participate in virologic surveillance for influenza and other respiratory viruses [3]. NREVSS, pronounced “nervous,” is a voluntary, laboratory-based surveillance system that monitors temporal and geographic patterns associated with the detection of respiratory syncytial virus (RSV), human parainfluenza viruses (PIV), respiratory and enteric adenoviruses (AdV) and rotaviruses [4]. NREVSS is a network consisted of collaborating university and community hospital laboratories, selected state and county public health laboratories, and commercial laboratories. NREVSS data are published periodically online at <http://www.cdc.gov/surveillance/nrevss/> and CDC's Morbidity and Mortality Weekly Report.

In the State of Maryland, the Department of Health and Mental Hygiene Laboratories Administration (MD DHMH Lab) Virus Isolation Lab has participated in NREVSS since the year of 2000. The Virus Isolation Laboratory test specimens for respiratory and enteric viruses throughout the year and routinely report virus isolation and antigen detection results to NREVSS. This network is an important part of the effort to prevent and control respiratory and enteric viral diseases in the State of Maryland.

Using the data we submitted to NREVSS from May 2009 to April 2010, we intend to identify respiratory viruses that are co-circulating with influenza viruses during the 2009 H1N1 influenza pandemic. We will describe the respiratory viruses of concern, including PIV, RSV, respiratory AdV, enterovirus and rhinovirus, examine the temporal patterns of these pathogens, and discuss the limitation and future direction of the respiratory virus surveillance system at the MD DHMH Lab.

2. Respiratory viruses

The viruses that commonly infect the respiratory tract and cause acute respiratory diseases in children and adults include the influenza A and B viruses, PIV type -1, -2, -3, RSV, AdV, and rhinoviruses. Viruses that principally target other organs but also infect the respiratory tract include the coxsackieviruses, echoviruses, and other enteroviruses. Table 1 summarizes the clinical symptoms and epidemiological features of the respiratory viruses that are currently

monitored in NREVSS. Respiratory tract infections include minor upper respiratory tract symptoms, namely common colds, bronchitis, flu, and flu-like illnesses, as well as more severe lower respiratory tract infections such as pneumonia and bronchiolitis. In addition to their ability to cause various respiratory illnesses, respiratory viruses share a relatively short incubation period and similar mode of person-to-person transmission by direct contact or aerosolized droplets. Respiratory infections represent a major public health problem because of their occurrence worldwide, rapid transmission in the community, and considerable morbidity and mortality.

Laboratory diagnosis of respiratory viral infections is traditionally performed by virus isolation in cell culture and serological tests such as hemagglutination inhibition tests, hemadsorption inhibition and immunofluorescence assays. Upper respiratory specimens such as nasopharyngeal aspirates or nasopharyngeal swabs, throat swabs are suitable for the detection of common respiratory viruses. For some respiratory viruses such as influenza and RSV, rapid diagnostic tests which detect minute amounts of viral antigen in infected cells present in respiratory secretions by using enzyme immunoassay have become widely available. These tests take about 15 to 20 minutes to complete and are usually limited to point of care setting because of their overall lower sensitivity and specificity when compared to cell culture or molecular assays.

Virus isolation in cell culture, although less commonly used in routine diagnosis, remains an important tool in epidemiologic and virologic studies. Respiratory viruses typically take several days to grow in traditional cell culture tubes, although rapid cell culture (centrifugation-enhanced shell vial) is available to detect specific viral antigens in 1 to 2 days. Direct fluorescent antibody staining of virus isolates or from clinical respiratory secretions can detect and identify six or seven respiratory pathogens. Nucleic acid amplification procedures including PCR, nucleic acid sequence-based amplification (NASBA), and loop-mediated isothermal amplification (LAMP) were developed for most respiratory viruses in recent years. Multiplex PCR coupled with fluidic microarrays using microbeads or DNA chips (oligonucleotides spotted onto a slide or chips) represents the latest diagnostic approach for the clinical laboratory and completes the evolution of diagnostics for respiratory viruses [5].

Table 1 Common viruses that cause respiratory tract infections (adapted from [6] and [7]).

Family	Genus	Virus and serotypes	Clinical symptoms	Epidemiologic features
<i>Orthomyxoviridae</i>	<i>Influenzavirus</i>	Influenza virus, types A and B	Sudden high fever, headache, myalgia, rhinitis, tracheobronchitis, pharyngitis, and cough	Seasonal influenza occurs as winter epidemics; Influenza A can cause pandemics
<i>Paramyxoviridae</i>	<i>Respirovirus</i>	Parainfluenza virus, types 1, 2, 3, 4A and 4B	Croup, rhinitis, pharyngitis, and bronchitis, often associated with fever	PIV-1 and PIV-2 can cause biennial fall outbreaks; PIV-3 occurs throughout the year, with peaks during the spring and early summer months.
	<i>Pneumovirus</i>	Respiratory Syncytial Virus, subgroups A and B	Cough, fever, sneezing, wheezing, hyperventilation, rhonchi, fine rales, and otitis media	Epidemics of RSV generally occur during fall, winter, and early spring in temperate climates
<i>Adenoviridae</i>	<i>Adenovirus</i>	Adenovirus serotypes 1-49	Common cold syndrome, pneumonia, croup, and bronchitis	Outbreaks of AdV-associated respiratory disease are more common in the late winter, spring, and early summer, but AdV infections can occur throughout the year
<i>Picornaviridae</i>	<i>Rhinovirus</i>	Rhinoviruses 1-110	Common cold, exacerbation of asthma & otitis media	Rhinovirus infections occur throughout the year, with peaks in the fall and early spring in the temperate climates
	<i>Enterovirus</i>	Coxsackieviruses A1-A24 (no 23), B1-B6	Nonspecific febrile illnesses, rashes, upper respiratory tract disease, and aseptic meningitis	Can produce a range of undifferentiated febrile illnesses, depending on the species and subtypes
		Echoviruses 1-34 (no 8, 10, 22 or 28)		
Enteroviruses 68-71				

2.1 Influenza virus

Influenza viruses comprise three types, A, B, and C. Influenza A virus was isolated in ferrets 1933; influenza B virus was isolated in 1940; influenza C virus was isolated in 1951. Only influenza A and B viruses are clinically important because influenza C virus infection occurs uncommonly [6]. Influenza A viruses causes moderate to severe illness in humans and animals (birds, swine, horses). It affects all age groups and undergoes frequent genetic mutations. Of all viruses that infect the respiratory tract, influenza A viruses cause the predominant number of serious acute respiratory tract illnesses, typically the flu and pneumonia [8].

Influenza A and B viruses belong to the family *Orthomyxoviridae* and the genus *Influenzavirus*. Their genomes are eight single-stranded, negative-sense segmented RNA. Their virions are enveloped particles about 100nm in diameter. The pleomorphic spherical virions contain several structural proteins, including the matrix protein, the nucleocapsid protein, and three RNA polymerase complex proteins, and two membrane glycoproteins, namely hemagglutinin (HA or H) and neuraminidase (NA or N). Mutations of the genes that encode HA and NA cause antigenic drift, resulting in variations in the antigenic characteristics of HA and NA glycoproteins. On the other hand, antigenic shift due to genetic reassortment of HA and NA can occur in cells infected with two different strains of influenza A viruses and can cause major changes in HA producing a new viral subtype. Antigenic variation enables the novel viral subtype to infect people whose antibody fails to recognize the new antigenic components of the virus. Influenza A and B undergo antigenic drift, and such variation is responsible for the development of seasonal epidemics. Only influenza A undergoes antigenic shift, unpredictably every 10-40 years, thus influenza A viruses have the potential to cause pandemics [7].

Influenza is characterized by abrupt onset of fever, sore throat, cough, headache, myalgia, and malaise. In uncomplicated cases, the illness usually abates in about 1 week, but the cough and weakness may sometimes persist for another week or more. Complications, such as secondary bacterial pneumonia and exacerbation of underlying medical conditions, are common in the very young children, the elderly and persons with underlying cardiovascular or pulmonary diseases and diabetes. Two subtypes of seasonal influenza A, H1N1 and H3N2, and influenza B viruses cocirculate hitherto in the human populations [9]. In the Northern hemisphere, seasonal influenza occurs as winter epidemics whereas influenza can occur throughout the year in the tropics. The timing and duration of flu seasons vary. While flu outbreaks can happen as early as October, most of the time influenza activity peaks in January or later.

2.1.1 2009 A (H1N1) pandemic influenza

Since mid-April 2009, a novel influenza A H1N1 virus has spread globally and caused pandemic influenza. The virus contains a unique combination of gene segments that previously has not been reported among swine or human influenza viruses in the United States or elsewhere, thus a large proportion of the population are susceptible to infection [10]. Since recognition of the novel influenza A (H1N1) virus in Mexico and the United States, the World Health Organization (WHO) raised the level of pandemic alert from phase 4 to phase 5 on April 29, 2009, indicating that human-to-human transmission of the virus had occurred in at least two countries in one WHO region [11].

This virus quickly spread across the globe. On June 11, 2009, WHO raised the level of influenza pandemic alert from phase 5 to phase 6, and declared that a global pandemic of H1N1 influenza was underway [12]. At the 1-year anniversary of the 2009 H1N1 influenza outbreak, as of April 25, 2010, worldwide more than 214 countries and overseas territories or communities have reported laboratory confirmed cases of pandemic influenza H1N1 2009, including over 17919 deaths [13].

The 2009 A (H1N1) virus contains a novel combination of gene segments that previously has not been recognized in swine or human influenza viruses in the United States or elsewhere, therefore immunity to the seasonal H1N1 virus do not protect against the pandemic virus. Phylogenetic analyses of this virus revealed that the HA, NP and nonstructural genes arise from the classical swine H1N1 lineage, the NA and M genes from the avian-like Eurasian swine H1N1 lineage while the PB2, PB1 and PA are from the North America H3N2 triple reassortant lineage [14].

The 2009 A (H1N1) infections can cause illnesses similar to seasonal influenza, including one or more of these clinical symptoms: fever, cough, sore throat, runny or stuffy nose, headache, body aches, fatigue, and sometimes vomiting and diarrhea. The 2009 H1N1 impacts younger adults and children more than older adults compared to seasonal flu. Moreover, most of the deaths caused by the pandemic influenza have occurred among younger people, including those who were otherwise healthy. Pregnant women, younger children and people of any age with certain chronic lung or other medical conditions appear to be at higher risk of more complicated or severe illness. Many of the severe cases have been due to viral pneumonia, which is harder to treat than bacterial pneumonias usually associated with seasonal influenza. Another key difference of 2009 A (H1N1) virus and seasonal influenza virus is its deviant temporal pattern. Unlike typical seasonal flu patterns, the new virus caused high levels of summer infections in the northern hemisphere, and then even higher levels of activity during cooler months in this part of the world.

2.2 Human parainfluenza virus (PIV)

First isolated from humans in 1956, PIV belongs to the genus *Respirovirus* in the family *Paramyxoviridae*. The paramyxoviruses range from 150 to 300 nm or more in diameter and are enclosed by a fragile lipid envelope. Similar to

orthomyxoviruses, the paramyxovirus are single-stranded negative-sense RNA viruses that possess fusion and hemagglutinin-neuraminidase glycoprotein on their surface. In contrast to the genome of orthomyxoviruses, the paramyxovirus genome is not segmented.

PIVs are comprised of five serotypes: types 1, 2, 3, 4A, and 4B. PIV-1, -2, and -3 occur worldwide and among persons of all age groups; PIV-4A and -4B are much less prevalent. Each of the four PIVs has different clinical and epidemiologic features. PIV-1 and PIV-2 cause croup illnesses (laryngotracheobronchitis) in infants and young children. PIV-3 is the second leading cause of pneumonia and bronchiolitis in infants and young children [6]. Among adults, PIVs can cause repeated infections with mild symptoms of minor upper respiratory tract illnesses (rhinorrhea, sore throat, and cough). PIVs can also cause serious lower respiratory tract disease with repeat infection (e.g., pneumonia, bronchitis, and bronchiolitis), especially among the elderly, and among patients with compromised immune systems.

PIVs are ubiquitous and infect most people during childhood. The highest rates of serious PIV illnesses occur among young children. PIV-1 causes biennial outbreaks of croup in the fall season in alternate years (usually during odd-numbered years in the United States). PIV-2 occurs more sporadically and can cause annual or biennial fall outbreaks. PIV-3 occurs throughout the year, although its activity peaks during the spring and early summer months each year.

2.3 Respiratory syncytial virus (RSV)

RSV, the most common respiratory pathogen of childhood, was first isolated in 1956. RSV is the most important etiological agent of serious lower respiratory tract disease, especially bronchiolitis and pneumonia, in children under 1 year of age in the United States [6]. Similar to PIV, which also belongs to the family *Paramyxoviridae*, RSV is an enveloped virus with a single-stranded, negative-polarity, non-segmented RNA virus. On the basis of reactivity with monoclonal antibodies, RSV can be differentiated into two subgroups, A and B (B1 and B2).

Symptoms of RSV infection are similar to other respiratory infections. In children, when the lower respiratory tract becomes involved (about 25 to 40% of infections), the child can develop a cough, fever, sneezing, wheezing, hyperventilation, rhonchi, fine rales, and otitis media [6]. In very young infants, irritability, decreased activity, and breathing difficulties may be the only symptoms of infection. In older children and adults, RSV can occur as reinfections against a background of partial immunity. The disease resembles a cold, with or without cough and fever. Most otherwise healthy individuals infected with RSV do not need to be hospitalized. In most cases, even among those who need to be hospitalized, hospitalization usually last a few days, and recovery from illness usually occurs in about 1 to 2 weeks. The elderly and adults with chronic heart or lung disease or with weakened immune systems are at high risk for developing severe RSV disease if reinfected.

Epidemics of RSV generally occur during fall, winter, and early spring in temperate climates. The timing and severity of RSV circulation in a given community can vary from year to year. In the northern temperate zones, the peak of the epidemic is usually January to April; and in the tropics, it is during the rainy seasons. During an epidemic, one or more strains from both subgroups may cocirculate. This resembles the circulation pattern of influenza type B but differs from that of influenza A, in which one variant (or a very small number of variants) dominates the scene to the exclusion of all other strains.

2.4 Respiratory adenovirus (AdV)

AdV, isolated in 1953, are non-enveloped virions containing a linear, double-stranded DNA molecule associated with an inner protein core. The outer capsid is composed of icosahedral capsomers that one (or two) fiber-like projection protruding from each of the 12 vertices. Currently, 49 human AdV serotypes have been identified.

AdV most commonly cause respiratory illness; however, depending on the infecting serotype, they may also cause various other illnesses, such as gastroenteritis, conjunctivitis, cystitis (bladder infection), and rash illness. Symptoms of respiratory illness caused by AdV infection range from the common cold syndrome to pneumonia, croup, and bronchitis. In industrialized countries, adenoviruses cause 2 to 5% of all acute respiratory tract diseases, while types 1, 2, 3, 5, 7 account for 87% of adenovirus infections [6]. Types 3, 4, 7, 14, and 21 may cause epidemics of acute respiratory tract infection among children and military recruits. Young infants and especially patients with compromised immune systems are more susceptible to severe complications of AdV infections, particularly those caused by types 7 and 3.

AdV infections occur worldwide, although epidemiologic characteristics of the adenoviruses vary by type. The fecal-oral route is the most common transmission of infection of AdV. AdV can also spread via aerosol droplets or direct contact in certain serotypes, thus causing respiratory illnesses in children and adults such as military recruits. Outbreaks of adenovirus-associated respiratory disease have been more common in the late winter, spring, and early summer; however, adenovirus infections can occur throughout the year.

2.5 Rhinovirus

Isolated in 1956 from patients with upper respiratory tract symptoms, rhinoviruses comprise a group of more than 100 antigenically distinct serotypes. Rhinoviruses cause 50% of all common colds, more than any other respiratory viruses [15]. Rhinoviruses belong to the family *Picornaviridae*, which also includes polioviruses, hepatitis A virus, and enteroviruses. The picornavirus virion is a naked icosahedron about 30nm in diameter. Its genome is a single linear molecule of single-stranded, positive-sense RNA that encodes four structural proteins and several nonstructural proteins.

Symptoms of the common cold include rhinorrhea, nasal congestion, sneezing sore throat, cough, and quite often a headache. There is little or no fever. The illness usually resolves within a week, but sinusitis and otitis media may supervene in infants and children [16]. Although rhinoviruses predominantly infect the upper respiratory tract, rhinovirus infections have been implicated in exacerbations of asthma and chronic bronchitis, and may induce acute lower respiratory tract disease, especially in young children, elderly persons, patients with chronic pulmonary disease and immuno-compromised individuals [17].

Rhinovirus infections occur throughout the year, with peaks in the fall and early spring in the temperate climates, and they occur during the rainy period in the tropics. Similar to other respiratory viruses, rhinoviruses also spread from person to person by direct contact and by aerosolized droplets.

2.6 Enterovirus

Enteroviruses are small, non-enveloped RNA viruses that also belong to the family *Picornaviridae*. Similar to the rhinoviruses, the enterovirus genome is a single molecule of single-stranded, positive-sense RNA. Enteroviruses, in contrast to the acid-labile rhinoviruses, are resistant to acidic pH, several proteolytic enzymes and bile salts, allowing them to inhabit the human alimentary tract. The *Enterovirus* genus contains multiple human pathogens including polioviruses, coxsackievirus groups A and B, echoviruses, and enteroviruses 68 to 71. Because the distinction between coxsackieviruses and echoviruses is now considered trivial, such nomenclature was abandoned and all enteroviruses identified since 197- have been allocated an enterovirus number, beginning with enterovirus 68 [7].

Infections with enteroviruses are very common and they are often subclinical. Nonetheless, they can cause a wide range of clinical syndromes involving many of the body systems including neurological disease, cardiac and muscular diseases, skin and mucosae conditions, and ocular diseases. Coxsackieviruses, echoviruses, and enteroviruses 68 to 71 have a less-specific extraintestinal target organ range than does poliovirus and therefore can lead to a wide range of illnesses. Coxsackieviruses and echoviruses, besides the alimentary tract, can infect the meninges, the central nervous system, myocardium and pericardium, striated muscles, respiratory tract, eye, and skin [6]. Although enteroviruses are not major causes of respiratory illnesses but can produce a range of undifferentiated febrile illnesses during summer and autumn. Coxsackieviruses A21 and A24 and echoviruses 11 and 20 are prevalent respiratory pathogens; coxsackie B viruses and certain echoviruses are less common. Coxsackieviruses may cause pneumonia in children and adults as well as pneumonitis in infants. Children may additionally suffer from pneumonia and bronchiolitis due to enterovirus 68 and an influenza-like illness caused by enterovirus 71 [6].

3. Laboratory surveillance

3.1 Virologic surveillance algorithm

In response to the public health emergency of the 2009 H1N1 influenza pandemic, the MD DHMH Lab collaborates with more than 100 sentinel providers and laboratories who participate in the Maryland outpatient influenza surveillance network (ILINet) to expand Maryland's laboratory-based influenza surveillance effort. At the beginning of the outbreak on April 26, 2009, the MD DHMH Lab requested sentinel providers and laboratories to submit all influenza A positive specimens or isolates for confirmation and typing. In addition, the MD DHMH Lab processed and tested respiratory specimens from individuals who present influenza-like illness (ILI) and meet CDC's travel and/or occupational risk exposures to 2009 influenza A (H1N1) virus infection.

As the H1N1 influenza infection expands in the community during the first wave, the MD DHMH Lab revised its laboratory-based influenza testing program on May 6, 2009. Use of real-time reverse transcriptase-polymerase chain reaction (rRT-PCR) testing is important for surveillance in order to identify which influenza A subtypes (e.g. 2009 H1N1 versus seasonal H1N1 or H3N2 viruses) are circulating. Testing was prioritized to focus on patients who presented to health care providers participating in ILINet with an ILI (i.e. temperature >100°F AND cough or sore throat OR sepsis-like syndrome) as well as those who were most severely affected (hospitalized or dead patients for whom influenza infection was clinically suspected). Moreover, submitters could submit specimens from ILI patients working with most vulnerable (healthcare workers) as well as those being evaluated within cluster/outbreak investigation of respiratory diseases. Viral testing of above individuals should be obtained through nasopharyngeal or

throat swabs (Dacron or synthetic on plastic or wire shaft in viral transport media only) or respiratory secretions (regardless of rapid test result).

When the 2009 influenza A (H1N1) pandemic activity reached its peak in Maryland in late October, the MD DHMH Lab revised its influenza specimen submission guideline again to focus on public health surveillance priorities. The MD DHMH Lab tested all specimens that were associated with ILI related hospitalizations, deaths, and outbreak investigations, but established a surveillance quota for outpatient specimens. Sentinel laboratories and university/college health care providers who are in the ILINet Program were restricted to 5 specimens per week for outpatient surveillance. For non-sentinel health care providers and laboratories, the MD DHMH Lab accepted only those specimens from hospitalized and dead patients due to an ILI and limited numbers of representative specimens as part of an active outbreak investigation. Figure 1 depicts the public health laboratory surveillance algorithm used in Maryland since May 6, 2009.

3.2 Laboratory detection methods

For respiratory virus testing, nasopharyngeal secretion samples in Universal Transport Media (Diagnostic Hybrids Inc., Athens, OH, USA) are collected from patients with influenza-like illness (ILI) and submitted to the DHMH by more than 100 clinical labs and sentinel providers of the Maryland ILI Surveillance Network (ILINet). Specimens are first screened by RT-PCR for influenza viruses. Specimens with test results negative for influenza are forwarded to the Virus Isolation Lab for inoculation. Specimens are cultured at 33°C using R-Mix cells (Diagnostic Hybrids Inc.), primary monkey kidney cells (Diagnostic Hybrids Inc. and ViroMed Laboratories, Minnetonka, MN), and HEp-2 cells (Diagnostic Hybrids Inc.). Identification of adenovirus, influenza A and B, parainfluenza viruses (PIV) 1, 2, and 3, and respiratory syncytial virus (RSV) is achieved by direct fluorescent antibody testing, using D³ Ultra™ Respiratory Virus Screening & ID Kit (Diagnostic Hybrids Inc.). In addition, specimens suspected to be rhinoviruses are sent to the Enterovirus Diagnostic Laboratory at CDC for confirmation and further characterization by PCR.

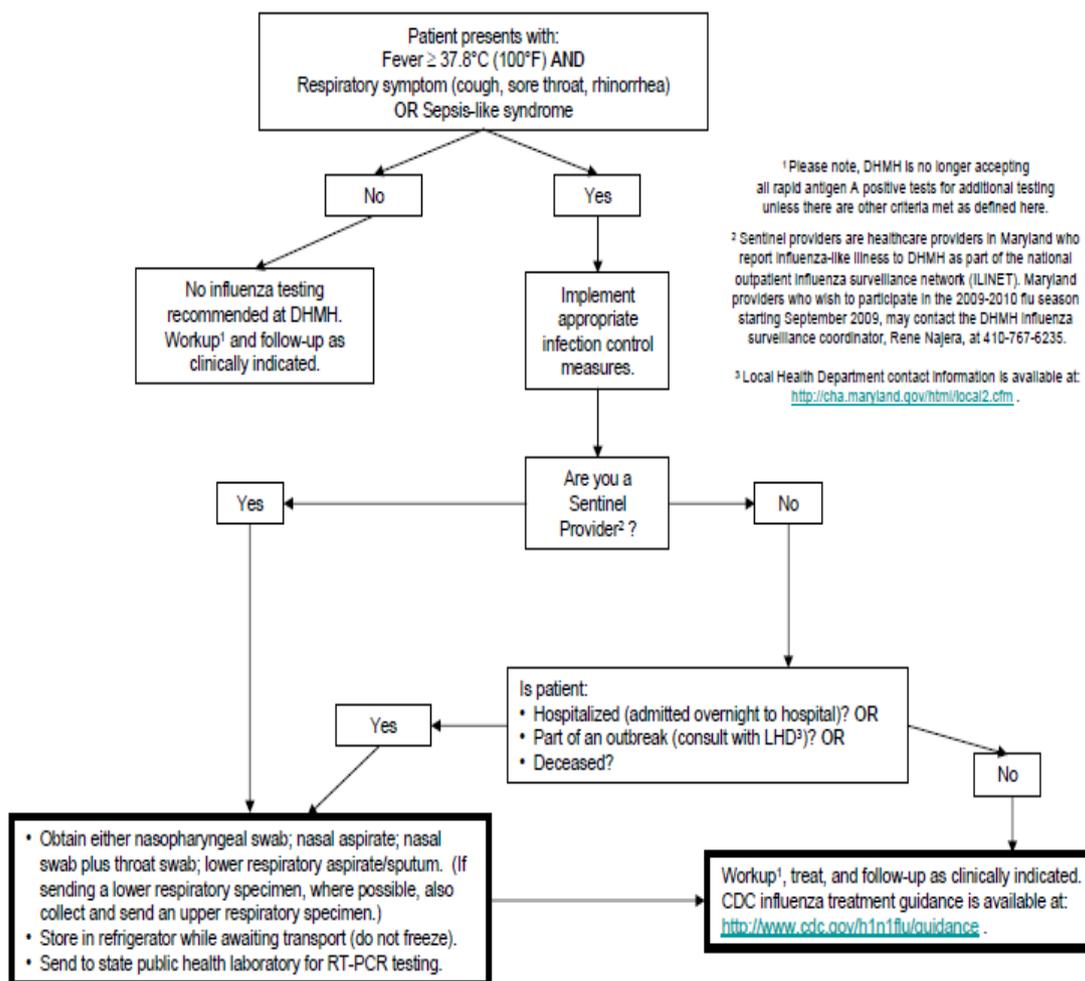


Fig. 1 Interim guidance for specimen collection for possible 2009 A (H1N1) influenza cases for providers and local health departments (LHD).

For enteroviruses, fecal specimens, cerebrospinal fluid and nasopharyngeal secretion samples are collected and inoculated at 37°C using E-Mix cells (Diagnostic Hybrids Inc.), RD cells (ViroMed Laboratories), WI-38 cells (Diagnostic Hybrids Inc.) and primary monkey kidney cells (either Diagnostic Hybrids Inc. or ViroMed Laboratories). Coxsackieviruses, echoviruses, and other enteroviruses are detected by indirect fluorescent antibody testing, using LIGHT DIAGNOSTICS™ Enterovirus Screening Set Kit (Millipore, Billerica, MA). Certain subtypes of coxsackieviruses (A9, A24, B1, B2, B3, B4, B5, and B6) and echoviruses (6, 9, 11, 30, and 4) are identified using LIGHT DIAGNOSTICS™ Coxsackievirus Antibody Set and LIGHT DIAGNOSTICS™ Coxsackievirus Antibody Set respectively. Specimens that cannot be subtyped (such as enteroviruses and other coxsackieviruses and echoviruses subtypes) in house are sent to the Enterovirus Diagnostic Laboratory at CDC for further characterization.

3.3 Surveillance data

From April 26, 2009 to April 24, 2010, 3312 specimens were inoculated in cell culture to test for non-influenza respiratory viruses and 161 specimens were inoculated to test for enteroviruses at the Virus Isolation Lab. As summarized in Table 2, the following non-influenza respiratory viruses were isolated: PIV (60), RSV (46), adenoviruses (36), and 22 viruses that belong to the rhinovirus or enterovirus group.

Table 2 Non-influenza respiratory viruses identified at the MD DHMH LABS during the 2009 H1N1 influenza pandemic.

Month Ending	Respiratory Virus by Virus Isolation				Respiratory and Enteric Virus by Antigen Detection	
	No. Tested	PIV	RSV	Adenovirus	Rhinovirus and Enterovirus	
		No. (%) Pos	No. (%) Pos	No. (%) Pos	No. Tested	No. (%) Pos
May 30, 2009	278	10 (3.6)	0 (0)	4 (1.4)	24	0 (0)
Jun 27, 2009	170	7 (4.12)	0 (0)	5 (2.9)	18	0 (0)
Jul 25, 2009	209	0 (0)	0 (0)	1 (0.5)	16	0 (0)
Aug 29, 2009	200	2 (1.0)	0 (0)	2 (1.0)	14	0 (0)
Sep 26, 2009	387	7 (1.8)	0 (0)	1 (0.3)	22	13 (60.1)
Oct 31, 2009	639	18 (2.8)	1 (0.2)	1 (0.2)	23	6 (26.1)
Nov 28, 2009	580	7 (1.2)	8 (1.4)	8 (1.4)	28	0 (0)
Dec 26, 2009	284	2 (0.7)	4 (1.4)	1 (0.4)	16	2 (12.5)
Jan 30, 2010	275	1 (0.36)	21 (7.6)	4 (1.5)	12	1 (8.3)
Feb 27, 2010	124	2 (1.6)	11 (8.9)	4 (3.2)	9	0 (0)
Mar 27, 2010	101	0 (0)	1 (1.0)	2 (2.0)	9	0 (0)
Apr 24, 2010	65	4 (6.2)	0 (0)	3 (4.6)	1	0 (0)
Total	3312	60 (1.8)	46 (1.4)	36 (1.1)	192	22 (11.5)

PIV represented the biggest group of non-influenza respiratory viruses the MD DHMH Lab identified during the influenza A (H1N1) pandemic. From April 2009 to April 2010, 24 PIV-1, 20 PIV-2, and 16 PIV-3 were isolated (PIV 4 is not detected in our current methodology). Activities of PIV-1 and PIV-2 peaked in late September and October 2009, with 11 PIV-1 and 7 PIV-2 isolated in that month. PIV-3 activity increased in the early summer months, and we isolated 7 PIV-3 in May 2009 and 5 PIV-3 in June 2009. RSV activities occurred concurrently with the annual seasonal influenza pattern with peak activity observed in January 2010. Adenoviruses were detected throughout the year but we were not able to subtype in our current surveillance effort.

We also isolated the following viruses that belong to the *Picornaviridae* family: rhinoviruses (6), coxsackieviruses (9), echoviruses (3), and enteroviruses (4). Rhinoviruses were only detected in September 2009 (3) and in January 2010 (1). Further characterization of rhinoviruses by PCR was performed by the CDC Enterovirus Diagnostic Laboratory. The rhinoviruses isolated in September 2009 were identified as rhinovirus 68 and two rhinovirus 1B; the one rhinovirus isolated in January 2010 was also rhinovirus 1B. Coxsackieviruses, echoviruses, and other enteroviruses all belong to the *Enterovirus* genus. Among the coxsackieviruses isolated, one was coxsackievirus B1; one was B2; five were B4; and two were B5. Among the echoviruses, one was echovirus 9 and two were echovirus 30. All enteroviruses were further characterized by CDC Enterovirus Diagnostic Laboratory and they were all enterovirus 68.

Compared to same period from May 2008 to April 2009, in which only 789 specimens were inoculated for respiratory virus isolation, the testing capacity at the MD DHMH Lab Virus Isolation Laboratory increased more than four-folds as a result of expanded laboratory surveillance effort for respiratory virus infections. Moreover, the percent positive rate by using virus isolation for PIV detection also doubled from 0.8% in May 2008-April 2009 to 1.8% in May 2009-April 2010. The percent positive rate for RSV and AdV isolation were consistent. For rhinoviruses and

enteroviruses, the percent positive rate by using antigen detection increased almost four-folds from 2.8% in May 2008-April 2009 to 11.5% in May 2009-April 2010.

3.3 Temporal patterns of respiratory viruses

Human PIV ranks among the most important groups of respiratory viruses that can cause acute upper and lower respiratory tract disease in infants and children. Each of the four PIV serotypes has different temporal pattern. PIV 1 causes biennial outbreaks of croup in the fall; PIV 2 causes annual or biennial fall outbreaks; and PIV 3 peak activity occurs during the spring and early summer months each year, but the virus can be isolated throughout the year. The activities of PIV monitored in Maryland were consistent with the epidemiologic features described above and also closely match the national trends reported in NREVSS. According to NREVSS, PIV-1 activity was elevated from early summer 2009 to early spring 2010, while PIV-2 activity was slightly increased in the fall of 2009 [18]. Our laboratory surveillance data indicated that PIV-1 and -2 activities surged in October 2009. The national trend of PIV-3 activity showed an increase in the spring and summer of 2009 [18]. The duration of PIV-3 activity in Maryland was less extended and was clustered in May and June 2009.

RSV represents the second largest group of non-influenza respiratory viruses isolated at the MD DHMH Lab during the 2009 A (H1N1) pandemic. In temperate climates, the RSV season generally begins during the fall and continues through the winter and spring, but the exact timing of RSV circulation varies by geographical location and year [19]. Outbreaks of RSV usually occur in communities each year, usually lasting 3–4 months during the fall, winter, and/or spring months. Our laboratory surveillance data indicated that the RSV season of 2009-2010 started in November 2009 and ended in March 2010 with peak activity in January 2010.

Outbreaks of adenovirus-associated respiratory disease have been more common in the late winter, spring, and early summer nationally; however, adenovirus infections can occur throughout the year. Our laboratory surveillance data indicated that adenoviruses were detected in the communities throughout May 2009 to April 2010. The overall level of adenovirus activities observed at the MD DHMH Lab was consistent with the national trend of continuously circulation of adenoviruses [20].

Rhinoviruses are commonly present in the community and rhinovirus infections occur year-round. However, our laboratory only identified 4 rhinovirus isolates from May 2009 to April 2010. Therefore our laboratory surveillance data did not describe the full extent of their circulation. Because rhinoviruses present mild or silent symptoms and most patients with the common cold may elect to stay home instead of visiting a clinic/hospital and getting laboratory diagnostic tests.

In the United States, infections caused by the enteroviruses are most likely to occur during the summer and fall. Our lab data indicated activities of coxsackieviruses, echoviruses, and enteroviruses mainly occurred in September and October 2009. It should be noted that enteroviruses are second only to the “common cold” viruses, the rhinoviruses, as the most common viral infectious agents in humans [6]. Although infections with enteroviruses are very common, most infections are silent, mild, or subclinical. Therefore, the small number of enteroviruses we were able to isolate is likely due to asymptomatic infections.

3.4 Limitation and future direction for respiratory virus surveillance at the MD DHMH Lab

Laboratory diagnoses of respiratory tract infections by the MD DHMH Lab not only provide active virologic surveillance in Maryland, but also support etiological diagnosis in outbreak investigations. However, respiratory virus detection and diagnosis is complex because of the wide range of pathogens which can present with the same clinical symptoms. Therefore, laboratory diagnostic and surveillance system should be strategized to provide information on viral epidemiology as well as provide clinical information.

The findings in this report, especially the laboratory surveillance data submitted by the MD DHMH Lab to the NREVSS, are subject to at least two limitations. First, the surveillance system relies on voluntary specimen submission and reporting, and the findings may not represent a comprehensive picture of virus circulating in the community. However, analyses of seasonality of several respiratory viruses (e.g. RSV) aforementioned have shown a correlation between our laboratory surveillance data and the national or regional trend observed in the NREVSS. For example, demographic or clinical information are often incomplete when submitters submit their respiratory specimens to us. Second, our laboratory respiratory virus surveillance system is based on isolation of viruses in cell culture. Although Direct fluorescent antigen detection (DFA) testing and cell culture are the traditional gold standard diagnostic tests for common respiratory viruses [21]. However, this methodology is not very sensitive and often negative for many picornaviruses, coronaviruses, human metapneumoviruses (hMPV) and human bocaviruses (hBoV) which are all recognized causes of respiratory symptomology and diseases in the communities [22]. Only 164 virus isolates were identified from the more than 3000 specimens cultured from May 2009 to April 2010 – an overall positive rate of 5.5%. A majority of specimens could not be successfully isolated due to a number of reasons including unsatisfactory specimen condition, sub-optimal sensitivity of culture-based methods, and a range of pathogens that are not currently part of our routine testing panel (e.g. PIV-4, hMPV, hBoV, and coronaviruses).

It has been recognized that molecular-based assays (nucleic acid amplification tests) have many advantages over antigen or culture-based methods including enhanced sensitivity, rapid turn-around time and wide range of pathogens detectable [22]. Simultaneous detection of a panel of respiratory viruses using multiplex PCR amplification and detection of products by suspension microarray is one of the most promising approaches for broad detection of respiratory viruses and improvement of viral surveillance [2, 23, 24]. For example, the xTAG Respiratory Viral Panel (RVP) from Luminex Molecular Diagnostics (Austin, Texas, US) allows for multiplex detection of 12 different respiratory viral targets using suspension microarray technology in the US and up to 20 targets outside the US [25]. Therefore our current testing algorithm may need to be reviewed to expand the testing panel using molecular assays, especially for viruses that could not be easily cultured.

4. Summary

Non-influenza respiratory viruses and enteroviruses are common in the communities and can cause respiratory symptoms similar to influenza virus infections. Although the H1N1 2009 pandemic influenza has dominated the scene this flu season, infections by other respiratory viruses and enteroviruses can easily add to the complication and confusion of clinical interpretation. Although, none of the above described viruses has been associated with an outbreak in Maryland this year, it is important to recognize and identify the activities of respiratory and enteric viruses circulating in our communities. Active surveillance of a wide range of respiratory and enteric viruses allows the continual monitoring of circulating viral strains in Maryland. The DHMH Laboratories Administration Virus Isolation Lab will continue to participate in the surveillance effort to prevent and control diseases caused by these pathogens.

Non-influenza respiratory virus and enterovirus infections can cause respiratory symptoms similar to influenza and can easily add to the complication and confusion of clinical interpretations. Viral surveillance and laboratory-based diagnostics are important to guide vaccine production, prophylaxis administration, and detection of novel viruses for certain respiratory viruses including influenza A [22]. Surveillance and early diagnosis have been shown to result in timely administration of antiviral drugs to decrease the duration of outbreaks and lower total costs due to illness; they also prevent the inappropriate use of antibiotics [26]. In addition, interactions between respiratory viruses can lead to changes in the circulation patterns and impact of different winter respiratory viruses [27]. These findings highlight the need to implement and continue to improve respiratory virus detection assays in addition to influenza testing for the clinical diagnosis and community surveillance of respiratory viral infections.

Acknowledgements I thank Dr. Maria Carlos, Dr. Robert Myers, and Dr. Jack Deboy for their patience, understanding, and continual support of my research effort and personal development. Especially thanks to all the members in the Virus Isolation Lab for their assistance, inspiration, and guidance. This research was supported in part by an appointment to the Emerging Infectious Disease (EID) Fellowship Program administered by the Association of Public Health Laboratories (APHL) and funded by the Centers for Disease Control and Prevention (CDC). Its contents are solely the responsibility of the researchers and do not necessarily represent the official views of the CDC.

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