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REVIEW ARTICLE

Diagnosis of influenza viruses with special reference to novel H1N1 2009 influenza virus

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Abstract On 15 April and 17 April 2009, novel swine-origin influenza A (H1N1) virus was identified in specimens obtained from two epidemiologically unlinked patients in the United States. The ongoing outbreak of novel H1N1 2009 influenza (swine influenza) has caused more than 3,99,232 laboratory confirmed cases of pandemic influenza H1N1 and over 4735 deaths globally. This novel 2009 influenza virus designated as H1N1 A/swine/California/04/2009 virus is not zoonotic swine flu and is transmitted from person to person and has higher transmissibility then that of seasonal influenza viruses. In India the novel H1N1 virus infection has been reported from all over the country. A total of 68,919 samples from clinically suspected persons have been tested for influenza A H1N1 across the country and 13,330 (18.9%) of them have been found positive with 427 deaths. At the All India Institute of Medical Sciences, New Delhi India, we tested 1096 clinical samples for the presence of novel H1N1 influenza virus and seasonal influenza viruses. Of these 1096 samples, 194 samples (17.7%) were positive for novel H1N1 influenza virus and 197 samples (18%) were positive for seasonal influenza viruses. During outbreaks of emerging infectious diseases accurate and rapid diagnosis is critical for minimizing further spread through timely implementation of appropriate vaccines and antiviral treatment. Since the symptoms of novel H1N1 influenza infection are not specific, laboratory confirmation of suspected cases is of prime importance.

Keywords H1N1 · Influenza virus · Influenza A

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Introduction

The current outbreak of swine influenza that originated in Mexico in March 2009 has spread to more than 80 countries causing more than 3,99,232 laboratory confirmed cases of pandemic influenza H1N1 globally and over 4735 deaths reported to World Health Organization (WHO)as of 11 October 2009 [1]. The WHO declared pandemic alert stage 6 on 11 June 2009, indicating an ongoing influenza pandemic [2]. The 2009 swine flu virus designated H1N1 A/swine/California/04/2009 is not zoonotic swine flu and is not transmitted from pigs to humans, but rather from person to person and has higher transmissibility than seasonal influenza viruses [3]. In humans, H1N1 swine flu presents as an influenza-like illness (ILI) with symptoms similar to seasonal influenza, i.e. fever, cough, sore throat, runny nose, muscle pains, severe headache, however, a considerable proportion of patients reported vomiting or diarrhea which is unusual in seasonal influenza [4, 5]. Since these symptoms are not specific to swine flu, early in the pandemic physicians were advised to consider swine influenza in the differential diagnosis of patients with acute febrile respiratory illness who had returned from Mexico or been in contact with persons with confirmed swine flu [6]. This new strain of H1N1 swine influenza has a unique combination of genes from both North American and Eurasian swine lineages that has not been identified previously in either swine or human populations [7]. The virus appears to be a result of reassortment of two swine influenza viruses, one from North America and one from Europe with the North American virus itself the product of previous re-assortments, carrying an avian PB2 gene for at least 10 years and a human PB1 gene since 1993. The virus also has genome segments of avian origin. Hence scientists call this novel strain as a "quadruple reassortant" virus. The hemagglutinin (HA)

gene is similar to that of swine flu viruses present in pigs in United States since 1999, where as neuraminidase (NA) and matrix (M) genes resemble viruses present in European pigs. Viruses with this genetic makeup have not previously been found in humans or pigs.

In India the novel H1N1 virus infection has been reported from all over the country. The most affected states are Maharshtra, Delhi, Tamil Nadu, Karnataka, Andhra Pradesh, Haryana, Kerala, Uttar Pradesh and Gujarat. As on 21 October 2009, a total of 68,919 samples from clinically suspected persons have been tested for influenza A H1N1 in government laboratories and a few private laboratories across the country and 13,330 (18.9%) of them have been found positive with 427 deaths [8].

The virus

Genomic analysis of the 2009 influenza A (H1N1) virus in humans indicates that it is closely related to reassortant swine influenza A viruses isolated in North America, Europe and Asia [Fig. 1] [9–11]. The segments coding for the polymerase complex, hemagglutinin, nuclear protein, and non-structural proteins show high similarity with the swine H1N2 influenza A viruses isolated in North America in the late 1990s. The segments coding for the neuraminidase and the matrix proteins of the new human H1N1 virus are, however, distantly related to swine viruses isolated in Europe in the early 1990s. In particular, the closest isolated relatives of the neuraminidase segment have 94.4% similarity at the nucleotide level with European swine influenza A virus strains from 1992 [11].

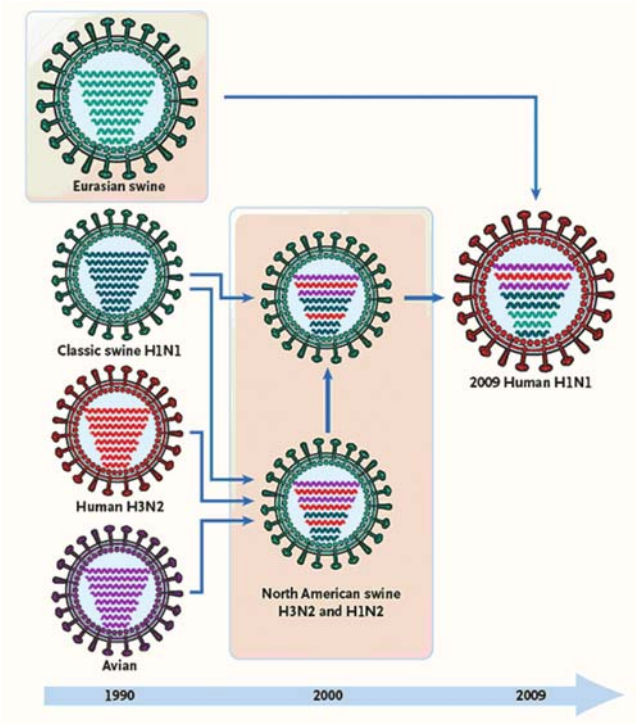

Fig. 1 History of reassortment events in the evolution of the 2009 influenza A (H1N1) virus. The segments of the human 2009 influenza A (H1N1) virus have co-existed in swine influenza A virus strains for more than 10 years. The ancestors of neuraminidase have not been observed for almost 20 years. The mixing vessel for the current reassortment is likely to be a swine host but remains unknown. (Modified from Vladimir T et al. 2009).

The incubation period for novel H1N1 2009 infection appears to range from 2 to 7 days; however, additional information is needed. On the basis of data regarding viral shedding from studies of seasonal influenza, most patients with novel H1N1 2009 infection might shed virus from 1 day before the onset of symptoms through 5 to 7 days after the onset of symptoms or until symptoms resolve; in young children and in immunocompromised or severely ill patients, the infectious period might be longer [12]. Patients who are at highest risk for severe complications of novel H1N1 2009 infection are likely to include but may not be limited to groups at highest risk for severe seasonal influenza: children under the age of 5 years, adults 65 years of age or older, children and adults of any age with underlying chronic medical conditions and pregnant women [13].

Treatment

Two classes of antiviral medication are available for the treatment of seasonal human influenza: neuraminidase inhibitors (oseltamivir and zanamivir) and adamantanes (rimantadine and amantadine). During the 2008–2009 influenza season, almost all circulating human influenza A (H1N1) viruses in the United States were resistant to oseltamivir [14]. However, genetic and phenotypic analyses indicate that novel H1N1 2009 is susceptible to oseltamivir and zanamivir but resistant to the adamantanes [15]. The Centers for Disease Control and Prevention (CDC), Atlanta, GA, USA has recommended that given the severity of illness observed among some patients with novel H1N1 2009 infection, therapy with neuraminidase inhibitors should be prioritized for hospitalized patients with suspected or confirmed novel H1N1 2009 infection and for patients who are at high risk for complications from seasonal influenza.

Diagnosis of novel H1N1 influenza virus

A number of different laboratory diagnostic tests can be used for detecting the presence of novel H1N1 influenza

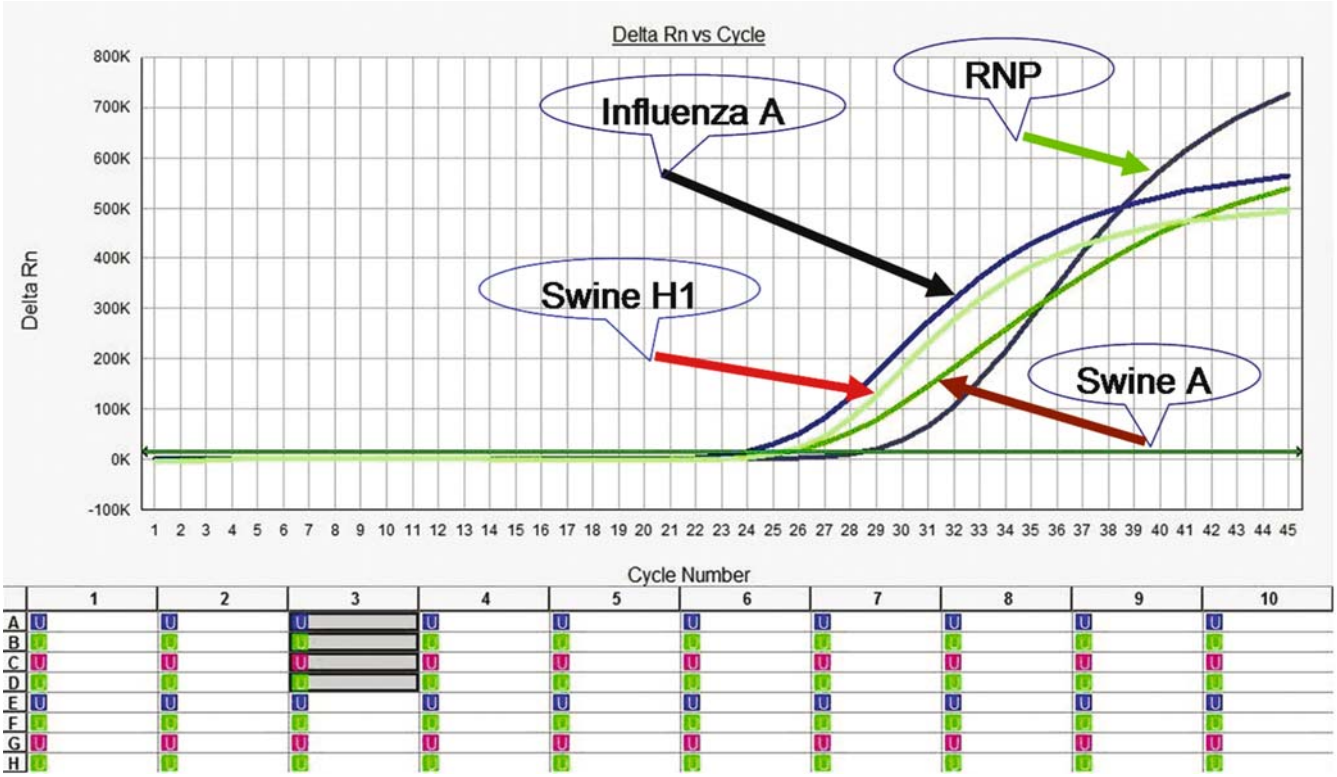

Fig. 2 Panel A. Real-time PCR amplification plot for novel H1N1 influenza virus detection. The grey highlighted area represents a sample in the 96 well reaction plate. Each sample is tested for seasonal Influenza A virus, novel H1N1 influenza virus A (Swine A), novel H1N1 influenza virus A subtype H1 (Swine H1) and internal control human RNaseP gene (RNP). The amplification plot for seasonal influenza virus A (black arrow), novel H1N1 influenza virus A (brown arrow), novel H1N1 influenza virus A subtype H1 (red arrow) and internal control RNP (green arrow) are shown in the figure.

virus in respiratory specimens, including direct antigen detection tests, virus isolation in cell culture, or detection of influenza-specific RNA by real-time reverse transcriptase-polymerase chain reaction (Real-time RT-PCR).

During outbreaks of emerging infectious diseases accurate and rapid diagnosis is critical for minimizing further spread through timely implementation of appropriate vaccines, antiviral treatment and prophylaxis where available and other public health-based non-pharmaceutical measures. Appropriate treatment of patients with respiratory illness depends on accurate and timely diagnosis and early diagnosis of influenza can reduce the inappropriate use of antibiotics and provide the option of using antiviral therapy.

Specimen collection

Preferred respiratory samples for influenza testing include nasopharyngeal or nasal swab, throat swab and nasal wash or aspirate, depending on which type of test is used. Samples should be collected within the first 4 days of illness. Routine serological testing for influenza requires paired acute and convalescent sera, does not provide results to help with clinical decision-making. Serological testing results for human influenza on a single serum specimen is not interpretable and is not recommended. All respiratory specimens should be kept at 4°C for no longer than 72 hours before testing and ideally should be tested within 24 hours of collection. If storage longer than 72 hours is necessary, clinical specimens should be stored at –70°C [16].

Antigen detection

Antigen detection tests; also known as rapid influenza diagnostic tests (RIDTs) detect influenza viral antigens in clinical specimens. These rapid influenza diagnostic tests can provide results within 30 min or less. Hence the results are available in a clinically relevant time period. Diagnostic tests for detection of novel H1N1 influenza virus antigen may be of two main types: direct fluorescent antibody (DFA) tests and rapid enzyme/optical immunoassays or assay for NA enzymatic activity.

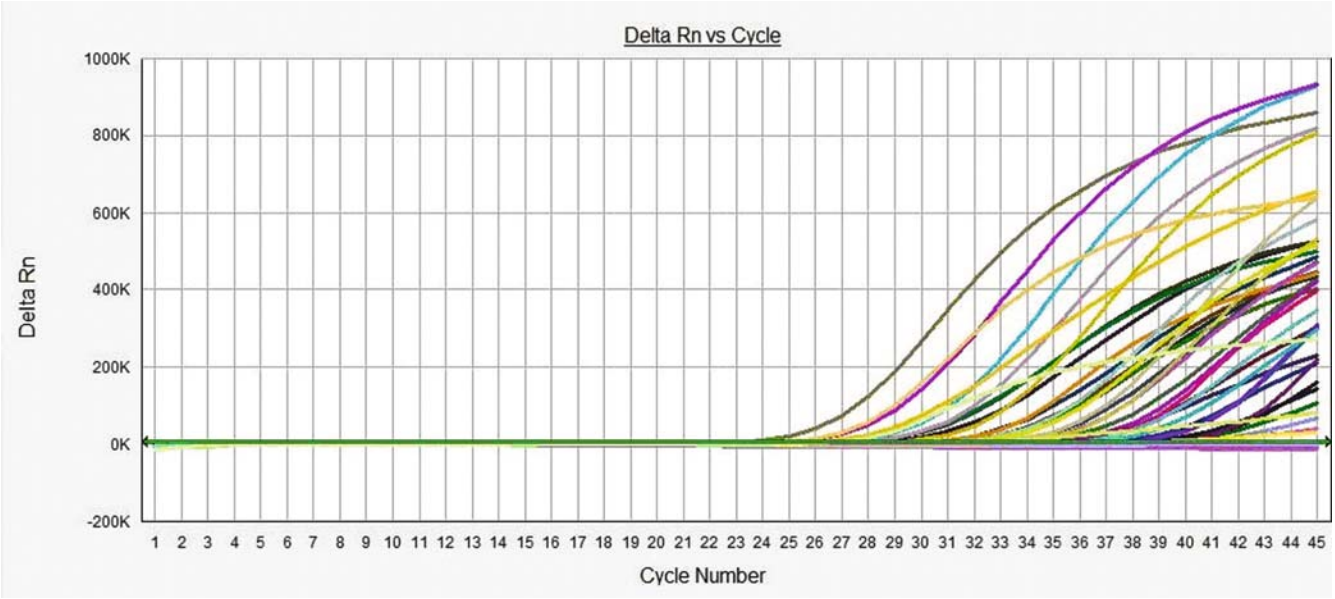

Fig. 2 Panel B. Real-time PCR amplification curve for novel H1N1 influenza virus detection in a 96 well plate format.

Direct fluorescent antibody (DFA) staining of clinical specimens using specific monoclonal antibodies against novel H1N1 influenza virus antigen can be a reliable and relatively rapid technique for the pandemic novel H1N1 influenza virus detection. Studies of DFA detection of influenza viruses have shown highly variable results with sensitivities ranging from 40% to 100%. Recent analytical studies indicate that commercially available RIDTs can detect novel influenza A (H1N1) virus [17]. In a study Chan et al. showed that the rapid antigen tests they evaluated in their study have comparable sensitivity for detection of novel H1N1 influenza and seasonal influenza viruses [18]. Data on analytical sensitivity for detection of different viruses does not directly reflect clinical sensitivity on patient specimens. However, only limited data have been published on the performance of RIDTs compared with RT-PCR for detecting the presence of novel influenza A (H1N1) virus in clinical specimens [19]. Compared to RT-PCR, the sensitivity of RIDTs for detecting novel influenza A (H1N1) virus infections ranged from 10% to 70%. The sensitivity of RIDTs to detect novel influenza A (H1N1) virus is equal to or lower than the sensitivity to detect seasonal influenza viruses [17]. Although these rapid tests do not differentiate between novel H1N1 2009 influenza virus and seasonal influenza A or even between subtypes H1 and H3 but they may provide useful information that might impact patient care. Understanding the limitations of rapid tests is very important to appropriately interpret results for clinical management of the disease [20].

Virus culture

Novel H1N1 influenza virus detection can also be achieved by inoculating the clinical specimen on MDCK cells for virus isolation with subsequent characterization by hemagglutination inhibition (HI) and neuraminidase inhibition tests using monospecific antiserum. Although the cell culture method is sensitive, it requires viable virus, needs expertise and at least 6–8 days to grow the virus to a level where cells are examined for cytopathic effect (CPE). Virus isolation is not only labor-intensive it is time-consuming also and requires a week for declaring a sample positive or negative hence not appropriate for an epidemic situation.

Molecular methods

Although the extreme genetic variability of influenza viruses is a challenge for design of molecular-based diagnostic tests. Reverse transcriptase-polymerase chain reaction (RT-PCR) is a widely used molecular tool that has been applied to both influenza virus detection and subtype characterization of virus isolates. Most influenza A PCR assays in use target conserved regions of the M gene and therefore should detect influenza A from all established subtypes, including the newly emergent novel H1N1 influenza. However, such methods need to be complemented with a rapid subtyping test to distinguish seasonal influenza A from novel H1N1 2009 influenza virus.

Table 1 Application of RT-PCR at AIIMS on clinical samples during novel A/H1N1 outbreak

Site	Total samples tested	Total +ve (%)	+ve seasonal influenza (%)	+ve Novel H1 N1 (%)
NICD	568	178(31.3)	129(22.7)	49(8.6)
AIIMS	528	215 (40.7)	68(12.8)	145(27.5)
Total	1096	393(35.9)	197(18)	194(17.7)

Multiplex PCR testing for the detection of respiratory viruses has seen major advances over the past decade resulting in the development of several commercially available tests. These tests can amplify one or more genes from a number of respiratory viruses and detect amplified products using microgene arrays. One such assay the xTAGTM RVP test was developed in 2005 immediately following SARS and H5N1 influenza and was designed to detect and type the three influenza A subtypes circulating at that time viz. H1, H3 and H5 [21, 22].

A limitation of PCR methods is that false-negative results may occur due to sequence variation in primer and probe targets and is particularly relevant for the detection of emerging viruses. However the use of multiple targets can reduce such limitations, and may serve as a means of confirming positive results. Mahony et al. recently demonstrated that the use of multiplex PCR tests such as the xTAGTM RVP test that use a combination of matrix and hemagglutinin gene targets can detect novel non-seasonal strains of influenza and hence can be used for the deection of novel H1N1 2009 influenza virus [23].

Real-time RT-PCR

RNA extraction

The efficiency and performance of nucleic acid amplification-based assays depends on the amount and quality of sample template. For detection of novel H1N1 2009 influenza virus validated and qualified RNA extraction, procedures should be used to ensure efficient recovery and purity. Commercially available extraction procedures including QIAamp® Viral RNA Mini Kit, or RNeasy® Mini Kit (QIAGEN), Roche MagNA Pure Compact RNA Isolation Kit, MagNA Pure LC RNA Isolation Kit II, and Roche MagNA Pure Total Nucleic Acid Kit have been shown to generate highly purified RNA following manufacturer's recommended procedures [24].

Nucleic acid amplification assays, including reverse transcriptase RT-PCR (rRT-PCR), and real-time RT-PCR are the most sensitive and specific influenza virus diagnostic assays. Real-time RT-PCR remains the method of choice for clinical diagnosis of novel H1N1 2009 virus in respiratory specimens and for differentiating it from seasonal influenza viruses [25]. Laboratory tests, such as real-time RT-PCR should be prioritized for hospitalized patients to diagnose 2009 H1N1 influenza and immunocompromised persons with suspected influenza where RIDT or DFA testing is negative or to determine influenza A virus subtype in patients who have died from suspected or confirmed influenza A virus infection.

The CDC has developed and recommended a real-time RT-PCR asaay for detection and characterization of novel H1N1 influenza. The assay includes a panel of oligonucleotide primers and dual-labeled hydrolysis (Taqman®) probes. The assay can be used to detect and characterize the novel H1N1 virus (swine influenza) in respiratory specimens and viral cultures. The assay has InfA primer and probe set designed for universal detection of type A influenza viruses and swInfA primer and probe set to specifically detect all swine influenza A viruses. The assay also includes a set of specific primer and probes for HA gene to specifically detect swine H1 influenza virus in specimens positive with SwInfA primers and probes. The assay can be applied on a wide range of specimens such as broncheoalveolar lavage, tracheal aspirates, sputum, nasopharyngeal or oropharyngeal aspirates or washes, and nasopharyngeal or oropharyngeal swabs taken from suspect swine influenza A infected patients. Recently Carr et al. developed an M gene-based real-time reverse transcriptase polymerase chain reaction (rtRT-PCR) assay for the detection of novel H1N1 2009 influenza virus that does not cross-react with human seasonal influenza A viruses (subtypes H1N1 and H3N2) [26].

Internal positive control

An internal control should be included for each and every clinical sample tested for novel H1N1 2009 virus. The inclusion of internal control ensures proper specimen collection, processing and RNA extraction. The CDC real-time RT-PCR protocol uses Human RNaseP gene (RNP) as internal control for human nucleic acids. No template

controls and positive template controls should also be included in each run. A human specimen control provides a secondary negative control that further validates the nucleic extraction procedure and reagent integrity.

Result interpretation

The no template control reactions should not exhibit fluorescence growth curves that cross the threshold line. The positive template control reactions should produce a positive result with the InfA, swInfA, swH1, and RNP reactions before 37 cycles. The human specimen control should not exhibit fluorescence growth curves except for RNP that cross the threshold line within 37 cycles. All clinical samples should exhibit RNP reaction curves that cross the threshold line at or before 37 cycles indicating the presence of sufficient RNA thus indicating the specimen is of acceptable quality. When all controls meet stated requirements, a specimen is considered presumptive positive for influenza A virus if the Inf A reaction growth curves cross the threshold line within 37 cycles. If the reaction for influenza A is positive, it may also be positive for universal swine and/or novel H1N1 2009 influenza. A specimen is considered presumptive positive for swine influenza A/H1 if both the InfA and the respective subtype (swInfA or swH1) reaction growth curves cross the threshold line within 37 cycles [24].

Although the real-time RT-PCR is highly sensitive and specific assay for novel H1N1 virus detection, the limitations include need of trained personnel for assay set up and result interpretation, false negative results which may occur if inadequate numbers of organisms are present in the specimen due to improper collection, transport, handling or excess of DNA/RNA template in the reaction and initial cost of machine.

H1N1 detections at the All India Institute of Medical Sciences

During this outbreak of novel H1N1 2009 influenza virus, at our facility in All India institute of Medical Sciences (AIIMS), New Delhi we tested a total of 1096 clinical specimens for the presence of novel H1N1 2009 virus and seasonal influenza viruses by real-time RT PCR, following CDC's protocol. Out of the 1096 samples, 568 samples were sent by National Institute of Communicable Diseases (NICD), New Delhi and 528 samples were from AIIMS. The specimens were nasal and throat swabs, transported under cold conditions to the virology laboratory. RNA was isolated using automated MagNA pure compact system (Roche Diagnostics) and subjected to real-time RT PCR. The positive, negative and internal controls were included in each run as per the protocol.

Of these 1096 samples, 194 samples (17.7 %) were positive for novel H1N1 influenza virus, 197 samples (18%) were positive for seasonal influenza virus. Out of the 568 samples tested from NICD, 49 samples (8.6%) were positive for novel H1N1 2009 influenza virus, 129 samples (22.7%) were positive for seasonal influenza viruses. From AIIMS 528 samples were tested, of these 145 samples (27.5%) were positive for novel H1N1 2009 influenza virus while 68 samples (12.8%) were positive for seasonal influenza viruses. Hence a total of 393 samples (35.9 %) samples were positive for influenza viruses (Table 1) (Fig. 2).

Summary

There is no perfect test for the diagnosis of influenza. Virus culture, the present 'gold-standard test' is not 100% sensitive and does not provide results in a time-frame that allows optimal use of potentially effective antiviral treatment. Although rapid diagnostic tests provide results in less than 30 minutes, they are significantly less sensitive and do not differentiate between different subtypes of influenza A virus. Rapid testing is only offered after the first culture-confirmed cases of influenza are reported from the community. Molecular assays; reverse transcriptase polymerase chain reaction (RT-PCR) and real-time RT-PCR targeting conserved regions of influenza virus genome have advantages over other methods and provide sensitive, highly specific and rapid diagnosis. The real-time RT-PCR should be the method of choice and both in-house developed and CDC-developed real-time PCR assays can be used for the specific detection of novel H1N1 2009 influenza virus.

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