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ORIGINAL ARTICLE

Diagnosis of Novel Pandemic Influenza Virus 2009 H1N1 in Hospitalized Patients

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Abstract A real-time RT-PCR assay was standardized and evaluated for the detection of the recent pandemic 2009 H1N1 strain that circulated around the world causing colossal loss of human life. We amplified the conserved regions of the hemagglutinin (HA) gene of 438 clinical specimens using real-time RT-PCR assay for rapid identification of pandemic influenza virus. The real-time RT-PCR was optimized and the primers and probes were tested against a panel of known negative and positive controls. RNA isolated from the HeLa cell line served as quality control. The conventional RT-PCR which is an established method of influenza virus diagnosis was compared to real-time RT-PCR. Of 438 clinical specimens tested, 212 specimens were found positive for influenza A virus (SD 46.669) in which 139 specimens were diagnosed positive for the pandemic 2009 H1N1 while 73 were the seasonal influenza viruses. We report that the real-time RT-PCR assay offers both, a high sensitivity and specificity when compared with the traditional identification method. The real-time RT-PCR assay allows rapid identification of the pandemic swine 2009-H1N1 at very low viral loads that are negative by the traditional RT-PCR. This optimized assay can be a very useful tool to assist both epidemiologists and the clinicians.

Keywords Swine influenza virus · Pandemic · Threshold cycle · Real-time quantitative PCR

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Introduction

Influenza A viruses are enveloped viruses with a segmented RNA genome consisting of eight single stranded RNAs of negative polarity [2]. These viruses are well known for the worldwide pandemics and epidemics [4, 8, 9] causing morbidity and mortality in individuals of all age groups, especially the elderly and the patients with chronic disabilities [5, 15]. The segmented nature of the viral genome permits gene reassortment events, which cause antigenic shifts for which there is no immunity in the population and also leads to development of new host range (facilitating pandemics) [6, 8]. After the three major pandemics during the twentieth century i.e. Spanish flu in 1918, Asian flu in 1957 and Hong Kong flu in 1968–1969 [7, 13, 14], the novel swine 2009-H1N1 virus has recently spread

internationally with such an unprecedented speed that the WHO escalated its pandemic alert from Phase 5 to Phase 6 on June 11, 2009 [16].

As of 22nd November 2009, this novel swine 2009-H1N1 virus has swept across almost all the continents of the globe, and has currently involved 207 countries and overseas territories/communities with over 6,22,482 laboratory confirmed cases including 7820 deaths [17]. India reported its first laboratory diagnosed swine H1N1 case in May 2009 while the first death due to 2009-H1NI was reported in August 2009 [9]. Control of the spread of disease relies on rapid diagnosis and appropriate clinical management. Early and reliable detection of the swine flu in clinical specimens will determine which patients, presenting with fever, should be immediately isolated and managed according to strict procedures of infection control. Several methods are available to diagnose influenza virus infection including rapid tests for the viral antigen present in the nasal and throat swabs or the naso-pharyngeal aspirates, immunofluorescence (IF) [1, 10] and virus RNA isolation followed by PCR. Real-time RT PCR is the latest gold standard for diagnosis of the novel pandemic swine 2009-H1N1 virus. Real-time RT-PCR assays for influenza virus using TaqMan-based probes have been recommended by WHO for the diagnosis of the currently circulating pandemic swine 2009-H1N1 virus [3].

In this study, a TaqMan based real-time RT-PCR assay for the rapid detection of pandemic swine 2009-H1N1 virus was evaluated using respiratory specimens. Results obtained from the real-time RT-PCR assay when compared to an established traditional RT-PCR assay revealed that approximately 13% of the specimens were detectable only by real-time RT-PCR.

Materials and Methods

Clinical Sample Collection

Nasal and throat swab samples were collected from four hundred and thirty-eight patients suspected of being infected with novel 2009-H1N1 virus. Samples were collected from various hospitals of Delhi (Kalawati Saran Children Hospital; Maulana Azad Medical College and Vishwanathan Chest Hospital), India and from the outbreak assistance laboratory (NCDC, Delhi) between the periods August to second week of December 2009. Most of the clinical specimens collected were from patients presenting with very high fever, running nose, sore throat and having close contact with a laboratory confirmed case and a recent travel history to places/countries with sustained human-to-human transmission of pandemic 2009-H1N1 influenza virus. All the clinical specimens (including nasal/throat swabs and nasopharyngeal aspirates) were carried in viral transport medium [11] and culture medium containing reference viral strain was used as the positive control. The Institutional Ethical Committee approved the study and informed consent was taken from the patients/Guardians before collection of samples.

RNA Extraction

The samples were handled in BSL-2 facility and viral RNA was extracted using the QIAamp viral RNA extraction kit (Qiagen) according to manufacturer's instruction [12]. Briefly, 500 µl of each sample was used for the extraction of viral RNA. The RNA was eluted from the columns with 30 µl Diethylpyrocarbonate (DEPC) treated water. The eluted RNA was used immediately or stored at −80°C for future use.

Determination of Lower Limit of Detection and Sensitivity of the Assays

The experiment was done with the viral RNA extracted from the swine H1N1 positive control sample. To exactly determine the lower limit of detection (LOD) and sensitivity of real-time RT-PCR assays & the conventional RT-PCR assays, serial dilutions of the extracted RNA, ranging from 2 ng to 2×10^{-7} ng, were used. Three replicates of each concentration were processed and analyzed by conventional RT-PCR and Real-time RT-PCR with pandemic 2009-H1N1 specific primers and probes (as per WHO recommendations).

Conventional RT-PCR Assay

Conventional RT-PCR for the detection of all type A and swine H1N1 influenza virus in the respiratory specimens were carried out according to the standardized protocol [16]. Conventional RT-PCR was performed on gradient PCR machine (Bio-Rad Laboratories) using Qiagen one-step RT-PCR kit according to the manufacturer's recommendations. Briefly, a 25 µl reaction mixture contained 5 µl 5× Qiagen RT-PCR Buffer, 1 µl dNTP mix (10 mM), 5 µl 5× Q solution, 1 µl of Enzyme mix, 0.25 µl of RNasin (20 U μl^{-1}), 1.5 µl (10 µM) each of forward and reverse primers and 2 µl of viral RNA and water to a final volume of 25 µl. The assay was based on the amplification of matrix gene (for all type A influenza viruses; Forward-Primer: 5′-TTCTA ACCGAGGTCGAAACG-3′ and Reverse-Primer: 5′-AC AAAGCGTCTACGCTGCAG-3′) [18] and of HA gene (for novel pandemic swine H1N1 viruses; HKU-SWF:5′-GAG CTCAGTGTCATCATTTGAA-3′ and HKU-SWR: 5′-TG CTGAGCTTTG GGTATGAA-3′) of influenza virus [19]. The reverse transcriptase step was carried at 50°C for

30 min., 95°C for 15 min. followed by 35 cycles of amplification (94°C for 30 s, 50°C (for M gene)/57°C (for HA gene) for 30 s, 72°C for 20 s), 72°C for 7 min and hold at 4°C. The PCR products were analyzed on 2% agarose gels and the amplicon size compared with the appropriate low molecular weight DNA ladder (Fermentas).

Real-Time RT-PCR Assay

Real-time RT-PCR reaction was carried out in a 25 µl mixture containing 5 µl RNA extract, 12.5 µl 2× reaction mix (SuperScript III Platinum One-step Quantitative RT-PCR kit, invitrogen), 0.5 µl (10 µM) each of forward primer and reverse primer, 0.5 µl (10 µM) probe and 1 µl of superscript III RT Taq Mix and 5 µl DEPC treated water. All primers and probes (Applied Biosystem) were provided by National Institute of Virology, Pune. The Inf A, sw A, swH1 and Rnase P primers and probe sets (Influenza A; Forward-Primer: 5′-GAC CRA TCC TGT CAC CTC TGA C-3′, Reverse-Primer: 5′-AGG GCA TTY TGG ACA AAK CGT CTA-3′, Probe: 5′-TGC AGT CCT CGC TCA CTG GGC ACG-3′, Swine inflenza A; Forward-Primer: 5′-GCA CGG TCA GCA CTT ATY CTR AG-3′, Reverse-Primer: 5′-GTG RGC TGG GTT TTC ATT TGG TC-3′, Probe: 5′-CYA CTG CAA GCC CA"T" ACA CAC AAG CAG GCA-3′, Swine H1; Forward-Primer: 5′-GTG CTA TAA ACA CCA GCC TYC CA-3′, Reverse-Primer: 5′-CGG GAT ATT CCT TAA TCC TGT RGC-3′, Probe: 5′-CA GAA TAT ACA "T"CC RGT CAC AAT TGG ARA A-3′ and Rnase P; Forward-Primer: 5′-AGA TTT GGA CCT GCG AGC G-3′, Reverse-Primer: 5′-GAG CGG CTG TCT CCA CAA GT-3′, Probe: 5′-TTC TGA CCT GAA GGC TCT GCG CG-3′) were provided to specifically detect type A influenza viruses, all swine influenza A viruses and the pandemic 2009-H1N1 viruses, respectively. The reaction was carried out in IQ cycler (Bio-Rad Laboratories) for 30 min at 50°C followed by 2 min at 95°C, with a subsequent 45 cycles of amplification (15 s at 95°C, 45 s at 55°C).

Results

Lower Limit of Detection and Sensitivity of the Assay

This technical specification indicates the concentration down to which an assay will detect the analyte with at least 95% probability. Each analyte concentration was tested in three replicate reactions in each RT-PCR assay and yielded a LOD of 2×10^{-3} ng RNA for the pandemic H1N1 influenza (HA) assay by conventional RT PCR and a LOD of 2×10^{-6} ng RNA ($\sim$2600 copies of RNA) by real time RT-PCR assay (Fig. 1 & Table 1). Compared to conventional RT-PCR assay, the real-time RT-PCR assay was around 1000 fold more sensitive for molecular detection of the viral RNA.

Detection of the Pandemic Swine H1N1 and Seasonal Influenza Virus in Clinical Samples

Samples corresponding to threshold cycle (Ct) value between 20 and 35 were considered to be positive for influenza A virus. Four hundred and thirty-eight samples were received and tested during the outbreak between August 2009 and second week of December 2009. Two hundred and twelve samples (48.40%) tested positive in the real-time RT-PCR assay, but only 186 (42.46%) tested positive by conventional RT-PCR (Fig. 2). 139 (31.73%) of 212 positive cases were positive for S-OIV and 73 (16.66%) were positive for seasonal influenza A virus.

Correlation of the Virus Infection with Age and Sex

The age group of 5–24 years was found to be most susceptible to swine 2009-H1N1 virus infection while the other age groups remained at comparatively lesser susceptibility. No specific pattern was seen in the age groups of the patients infected with seasonal influenza virus (Table 2). We observed that males were more susceptible to infection with pandemic swine 2009-H1N1 virus as compared to the female in the ratio of 1.641:1, while the ratio in case of the seasonal influenza viruses was 1.259:1 (suggestive of the exposure to infected areas).

Discussion

In this study, we demonstrate that the real-time RT-PCR assay, that specifically targets the hemagglutinin gene of the 2009-H1N1 influenza A virus, is more sensitive than the traditional RT-PCR assay. The primers and probes recommended by WHO is a reliable identification method used for the detection of the pandemic swine 2009-H1N1 virus in the clinical samples taken from the suspected patients. Unlike traditional RT-PCR, the real-time RT-PCR assay not only reduces the risk for contamination but also reduces turnaround time to 1–2 h. CT values of less than 25 showed a very high correlation with positive results obtained using the viral nucleic acid by the conventional PCR. Further, a Ct of less than 20 had a specificity that approached 100% for the swine 2009-H1N1 virus as well as seasonal influenza viruses. This indicates that the real-time RT-PCR assay can be used to reliably confirm the presence of influenza viruses in patients that test positive by traditional methods. In the current study, 48.40% of the samples tested positive by the real-time RT-PCR assay

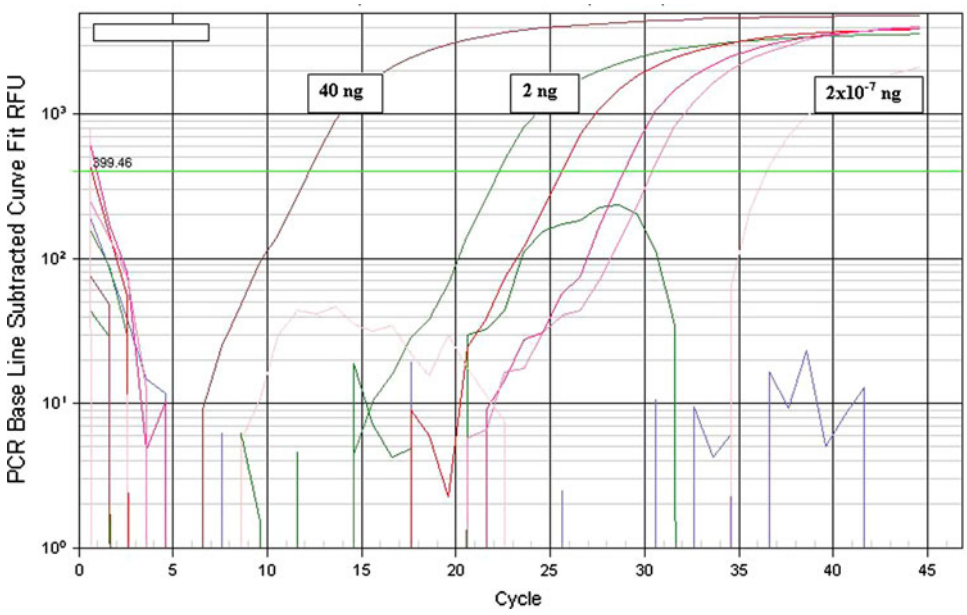

Fig. 1 Real-time RT-PCR analysis of different dilutions of the viral RNA: RNA was isolated from positive control sample for S-OIV. Eight 10 dilutions of this RNA, ranging between 2 ng and 2×10^{-7} ng were prepared and RT-PCR was performed using HA gene specific primers and probes

Table 1 Determination of the lower limit of detection of viral RNA with real-time and conventional RT-PCR

	Amount of RNA used as template in PCR reactions							
	2 ng	2×10^{-1} ng	2×10^{-2} ng	2×10^{-3} ng	2×10^{-4} ng	2×10^{-5} ng	2×10^{-6} ng	2×10^{-7} ng
Conventional RT PCR[a]	+	+	+	+	−	−	−	−
Real Time RT PCR[a]	+	+	+	+	+	+	+	−

[a] RT-PCR was performed with eight different dilutions of RNA, starting from 2 ng to 2×10^{-7} ng; (+): positive results for PCR; (−): negative result

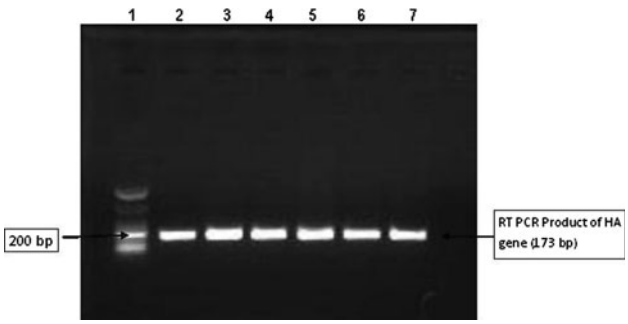

Fig. 2 Amplification of HA gene of the pandemic swine 2009-H1N1 by conventional RT-PCR: Lane 1: 100 bp DNA ladder. Lane 2–7: RT-PCR product of HA gene of swine 2009-H1N1 virus

while the traditional PCR detected only 42.46% thereby showing the superior sensitivity of real-time RT-PCR assay. The analysis of the patients found positive for swine 2009-H1N1 and seasonal influenza A revealed that patients in 5–24 years age group were more vulnerable to pandemic swine 2009-H1N1 virus while the seasonal influenza virus showed no specific pattern. The probable reason for less infection in the elderly population could be cross preventive effect of the antibodies developed against the circulating seasonal influenza A viruses. This argues that the vaccination of the youngsters from 5 to 24 age groups against the pandemic virus is indeed urgent. Another important observation was that males as opposed to females were found to be more susceptible with a ratio of 1.641:1 and 1.259:1 for swine 2009-H1N1 and seasonal influenza viruses, respectively.

Table 2 Age-wise distribution of the population found positive for S-OIV

Age groups (Year)	Virus	
	Swine influenza virus (H1N1) (%)	Seasonal influenza A virus (%)
0–4	14.38	16.43
5–24	42.44	28.76
25–50	14.38	31.50
51–60	2.87	6.84

In conclusion, a rapid, sensitive and specific real time RT-PCR assay is described for the detection of pandemic swine 2009-H1N1 viruses. This assay can be very useful for rapid and early diagnosis of the suspected patients. In summary, we can show that the real-time RT-PCR assay is very sensitive and can provide a highly useful technique for pandemic testing.

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