Comparative reproducibility of SYBR Green I and TaqMan real-time PCR chemistries for the analysis of matrix and hemagglutinin genes of Influenza A viruses

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Running title: Comparison of real-time PCR for detection of influenza virus.

ABSTRACT

Background: The outbreak of novel influenza A H1N1-2009 virus (pH1N1) and its rapid spread worldwide raised serious concern about pandemic preparedness.

Objectives: The present study was designed to evaluate the sensitivity and specificity of two chemistries of real-time RT-PCR (with the use of fluorescent *SYBR Green I* dye and specific *TaqMan* probe) for detection of the matrix and hemagglutinin genes of human influenza A viruses.

Methods: Influenza A virus reference strains were used to perform the calibration curve analysis and the diagnostic accuracy of both the real-time RT-PCR formats were assessed on 110 clinical specimens from patients presenting with influenza-like-illness (ILI).

Result: The study results suggest that the TaqMan chemistry has better sensitivity as compared to *SYBR Green I,* however, the *SYBR Green I* assay was found to be simpler, economical and readily available.

Conclusions: It is concluded that the SYBR Green I assay has equal potential to be considered as an alternative to the TaqMan assay as a preparedness measure for any future influenza outbreak management.

Keywords: Influenza virus, Real-time PCR, TaqMan assay, SYBR Green assay

Introduction

Influenza A viruses continue to source menace across the globe, causing thousands of deaths and enormous socio-economic loss[1-3]. They are therefore epidemiologically significant and among the most studied viruses with respect to the worldwide respiratory diseases[4]. Surveillance and early diagnosis of influenza is important in providing information concerning the circulating strains of the virus in a community. The emergence of the novel influenza A H1N1-2009 virus in humans has once again recognized the pandemic potential of the influenza A virus[5], engulfing 18449 individuals from over 214 countries and territories[6]. The recent pandemic due to H1N1-2009 virus was unique from the previous three major pandemics, i.e. "Spanish Flu" 1918-1919, "Asian Flu" 1957 and "Hong Kong Flu" 1968 as its spread was very rapid across the world[5]. The rapid rate of evolution of this virus and its high rate of transmissibility calls for highly sensitive and specific molecular tests for the rapid detection of influenza A viruses. Real-time quantitative PCR (qPCR) is an advanced technology for quantification of nucleic acids, gene expression studies and has become an essential need in diagnostic research works. The high precision, sensitivity and specificity of qPCR assays have led to development of 20 different chemistries, of which TaqMan and/or SYBR Green chemistries are the most widely used[7]. The TaqMan PCR exploits the 5′–3′ nuclease activity of Taq polymerase to cleave a sequence specific fluorescent labelled probe[8] while the SYBR Green assay is based on the principle of intercalating fluorescent dyes between the minor grooves of the amplified DNA during the primer annealing and extension steps of each PCR cycle. Owing to the high precision, sensitivity and specificity, the World Health Organization (WHO) recommended the use of the TaqMan chemistry[9] for detection of the pandemic H1N1 virus in the human respiratory samples during the pandemic period[10]. However, the use of TaqMan Chemistry is costly and not all virology laboratories could afford to use this PCR method thus limiting the number of laboratories designated for the pandemic H1N1 testing during the 2009-2010 pandemic period. The pandemic H1N1 virus almost replaced the seasonal influenza (H3N2 and H1N1) viruses with marked seasonality and in the post pandemic phase, it is still circulating in the community having potential of reassortment and production of new influenza virus strains of unpredictable transmissibility and infectivity.

During pandemic situations, when the number of suspected cases increases drastically, it becomes difficult to control the spread of pathogen and provide diagnostic results in time. Some times the cost of diagnostic tests becomes the limiting factor. In the present study, the

performance of two real-time PCR chemistries were compared to recognize the potential of the SYBR Green I chemistry as an alternative to the TaqMan assay for the specific detection of pandemic as well as seasonal influenza A viruses.

Material & Methods

Clinical specimen collection and virus propagation: Clinical specimens (nasal and/or throat swabs) were collected from 110 patients exhibiting fever and two or more symptoms of ARTI (cold/cough, running nose, sore throat, myalgia, body ache) from OPD of different hospitals of Delhi region, India, in a duration of two months (August to September 2010) during which influenza activity was at its peak. All clinical specimens were subjected to real-time RT-PCR analysis[11] for screening the pandemic H1N1-2009 viruses from the seasonal influenza A viruses in our laboratory. The hospitalized patients were not included in the study to rule out nosocomial infections. The PCR positive pandemic H1N1-2009 specimens were also sequenced commercially to confirm the source of their origin. The reference strains of influenza A virus (A/PR/8/34-H1N1 and A/Udorn/307/72-H3N2) procured from CDC, Atlanta, GA, USA, and the pandemic H1N1-2009 (A/Delhi/1/2009-H1N1) isolated at our laboratory, to serve as positive control, were propagated to sufficiently high titres in 10-days old embryonated chicken eggs and the virus-containing allantoic fluid was harvested and stored in -80 ºC until use. This study had necessary ethical clearance form the institute ethical committee (IEC) for clinical specimen collection and informed consent was taken from patients/guardians before collection of specimens.

RNA extraction and cDNA synthesis: Viral RNA was extracted from 250 µL of all the clinical specimens and from 140 µL of the virus-containing allantoic fluid (reference strains) using commercially available QIAamp Viral RNA Mini Kit (Qiagen GmbH, Hilden, Germany) as per manufacturer's instructions. cDNA was prepared using IM-PROM reverse transcription kit (Promega, Madison, WI, USA).

Real-time RT-PCR: Two different real-time RT-PCR assays, one with the fluorescent *SYBR Green I* dye and second with the specific *TaqMan* probe were used in the study. The reactions were performed for each clinical specimen and reference strains in triplicates and were regarded as positive when at least two out of the three wells confirmed positive results. For both the PCR assays, the positive controls were run in four 10-fold dilutions for the calibration curve analysis as described previously by our group[3].

Real-time RT-PCR using SYBR Green I: The real-time RT-PCR consisted of SYBR Green RT-PCR reaction mix (Bio-Rad Laboratories Inc.), 0.1 µM of each forward and reverse primers designed specifically to amplify the matrix (all type A) and HA gene (pH1N1) of influenza A viruses as per CDC protocol[11, 12], 5 µL of cDNA and RNAse free water upto a volume of 25 µL. The PCR thermal cycling was performed in an iCycler iQ5 realtime PCR detection system (Bio-Rad Corp., Hercules, CA, USA) with the following cycling conditions: 95 ºC for 5 min followed by 40 cycles at 95 ºC for 15 s and 55 ºC for 30 s. A melting curve analysis was performed after

the PCR cycling, where the temperature was increased by 0.5 ºC every 10 s, starting at a temperature of 55 ºC.

Real-time RT-PCR using TaqMan probe: The real-time RT-PCR consisted of Platinum Quantitative PCR SuperMix-UDG (Invitrogen, Carlsbad, CA, USA), 0.1 µM of each forward, reverse primers, that were used with the SYBR Green I assay, and probes designed specifically to amplify the matrix (all type A) and HA (pH1N1) genes of influenza A viruses[11, 12], 5 µL of cDNA and RNAse free water upto a volume of 25 µL. The reaction was carried out with the following cycling conditions: 95 ºC for 5 min followed by 40 cycles at 95 ºC for 15 s and 55 ºC for 30 s.

Conventional RT-PCR assay: All the clinical samples were also screened in parallel by a conventional RT-PCR assay for detection of the matrix gene for all type A and HA gene specific for the pandemic H1N1-2009 infections as described earlier[12] to assess the specificity of the real-time RT- PCR assay.

Statistical analysis: Statistical analysis was carried out using Prism (Version 5) software. All *P* values were two-tailed and $P \leq 0.05$ was considered significant.

Results

A total of 110 clinical specimens collected during the two months (August to September 2010), were analyzed for influenza A infections in two different real-time PCR chemistries; with SYBR Green I and TaqMan with specific probe. Of the 110 clinical specimens analyzed, a total of 70 (63.63%) were identified as influenza A positive by both the PCR chemistries with variable Ct values, of which the SYBR Green I could detect 24 (21.8%) samples as the pandemic H1N1 and 46 (41.8%) as seasonal influenza A viruses, while the TaqMan assay could detect 29 (26.36%) as pandemic H1N1 and 41 (37.27%) as the seasonal influenza A viruses. Six (5.45%) clinical specimens were found positive for pandemic H1N1 exclusively by SYBR Green I while they were negative for influenza viruses by the TaqMan assay. The Ct value for the pandemic H1N1 ranged between 20-35 (mean Ct ± SD, 28.46 ± 5.176) with SYBR Green I and 21-34 (mean Ct ± SD, 26.68 ± 4.246) with the TaqMan assay while the Ct values for seasonal influenza A ranged from 17-35 (mean Ct ± SD, 26.13 ± 5.414) and 18-34 (mean Ct ± SD, 24.35 ± 4.249) with the SYBR Green I and TaqMan assay respectively. The clinical specimens corresponding to Ct values between 20 and 35 were considered to be positive for influenza A virus. The RNase P gene was co-amplified with all the reactions to serve as an internal positive control for the human nucleic acids.

All the clinical samples, run in parallel, by the conventional RT-PCR detected 19 (17.27%) specimens as pandemic H1N1 while 34 (30.9%) as the seasonal influenza A viruses.

Discussion

Surveillance and epidemiological investigation of the respiratory viruses is an inevitable part of health care and disease prevention. The rapid diagnosis and treatment of any infection has been shown to be crucial for an outbreak control[13]. Recently, real-time PCR has been shown to be very useful in the detection of various pathogens in clinical specimens[14, 15]. It meets all the requirements for the analyses used in clinical practice: rapid analysis, automation, requirement of very small quantity of clinical specimens, reliability and reproducibility of the results. The primer sets and probe sequences used in real-time PCR provides an additional level of specificity as demonstrated earlier by our group[3, 12].

This is one of the very few reports regarding the comparative analysis of two important real-time PCR chemistries (SYBR Green I and TaqMan assay) for detection of human influenza viruses (pH1N1)[16]. In this present study we have reported the comparative results of the two real-time chemistries for highly sensitive and specific detection of the matrix and HA genes of human influenza A viruses. The TaqMan assay uses the fluorogenic probe to enable the specific detection of the PCR product while the SYBR Green assay uses a highly specific, double-stranded DNA binding dye, to detect PCR product. For real-time PCR, the TaqMan mode, which is of higher specificity, is used more widely than the SYBR Green I approach[17] however mutations within the probe-binding site can prevent annealing of the probe and subsequent detection[18]. The SYBR Green I mode requires two conserved regions for primer binding, whereas the TaqMan approach requires three conserved regions in the targeted sequences for primer and probe binding respectively.

Therefore, in principle, the SYBR Green I mode can be considered more appropriate for detection of a highly variable sequence, such as that of the HA gene of influenza viruses and this could be one of the possible reasons for the 6 clinical specimens that were detected positive only by SYBR Green I and negative by the TaqMan assay in our current study. A similar observation has also been documented for herpes simplex virus[19] and is a great concern for influenza viruses.

Overall the cost related to procurement of the TaqMan probe is much higher as compared to the SYBR Green I dye which makes the later popular in terms of economy and ease of availability. The sensitivity of both the chemistries are acceptable as observed in our results, however the specificity of SYBR Green may be misleading, in few cases, with variation in the amount of template used, resulting in an overestimation of the target concentration[20]. Owing to the greater benefits with this chemistry, it can be considered as an alternative to the only TaqMan chemistry available with us for pandemic H1N1 virus detection and for the effective management of any future outbreak situation.

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