Development of a sensitive real-time PCR for simultaneous detection and subtyping of influenza A and B viruses

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Abstract

A new real-time PCR assay, using melting curve analysis, was developed for the rapid and reliable detection and sub-typing of influenza A and B. In order to evaluate it's specificity, cell culture surnatants positive for Respiratory Syncytial Virus, Parainfluenza Viruses 1, 2 and 3, Measles Virus, Influenza A (to evaluate Influenza B primer) and B (to evaluate Influenza A primer) were tested and all of the results were negative. A series of Influenza A and B cell culture-grown viruses were diluted in virus transport medium, titrated and tested to determine the analytical sensibility which equated to 0.64, 0.026, 0.64, 0.62 PFU for A/H1N1, A/H3N2, Victoria-like and Yamagata-like B viruses, respectively. Twenty-five specimens, collected during the 2001/02 and 2002/03 seasons, which were positive for A/H1N1 (n = 7), A/H3N2 (n = 10), B Victoria-lineage (n = 5) and B Yamagata-lineage (n = 3), were tested in order to evaluate the assay's clinical sensitivity, all of the results were positive. The new real-time PCR appears to be a suitable tool for virological surveillance and the diagnosis of respiratory infections.

Key words: influenza viruses, real-time, virological surveillance

Introduction

Influenza viruses are a common cause of upper and lower respiratory tract disease and are responsible for morbidity and mortality especially in the elderly and in high risk groups such as patients with chronic diseases and those who are immunocompromised.[1-3]

The etiological diagnosis of an influenza like illness can be very difficult and the rapid and sensitive laboratory confirmation of clinically suspected influenza cases is useful in order to commence appropriate antiviral therapy, for epidemiological surveillance and for the control of influenza outbreaks in hospital and nursing home settings.[4-5]

Retrotranscriptase (RT)-Polymerase Chain Reaction (PCR) and nested RT-PCR are commonly used for typing and sub-typing influenza viruses as they show high sensitivity and specificity, but they are expensive, time-consuming and prone to the risk of cross-contamination. The aim of this study was to develop and evaluate a new real-time PCR for the detection and typing of influenza A and B viruses from respiratory samples, making the diagnostic procedure more rapid and reducing the risk of contamination. The new assay was designed to detect (i) influenza A, subtype H1N1 and H3N2, including the new A/H3N2/Hong Kong/1143/02-like variant, which appeared during the 2002-03 season predominated during the following seasons, and (ii) the two lineages of influenza B (Victoria/2/87 and Yamagata/16/88), that have been co-circulating since the 2001/02 season.[6-8]

Methods
Primers design

Primers were selected based on a highly conserved region, after the alignment of the nucleotide sequences. They were designed and chosen in a highly conserved region of the matrix protein for influenza A and in the hemoagglutinin (HA) for influenza B. Multi-alignments were used to select highly conserved regions using the MEGA package, version 1.01 from the Pennsylvania State University (PA, USA). Primers were manually designed in order to obtain homogeneous melting temperatures which were calculated using primer 3 software.[9] The primers sequence were as follows: for influenza A, FLUAMATR 10: 5' ATG GAA TGG CTA AAG ACA AGA CC 3' and FLUAMATR232 5' AAG TGC ACC
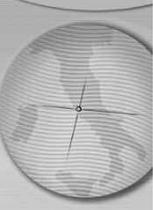
AGC AGA ATA ACT GAG 3', and for influenza B, FLUBHA115 : 5' TCT CAT TTT GCA AAT CTC AAA GG 3' and FLUBHA280 5' GTT CTG TCG TGC ATT ATA GGA AAG 3'.

RNA extraction

RNA was extracted from each sample using the QIAamp Viral RNA Mini spin protocol (Qiagen, Hilden, Germany), according to the manufacturer's instructions.

In brief, a sample of 250 μl was extracted and the nucleic acids were eluted in 30 μl of RNasi free water and stored at –80°C.

PCR Procedure

RT was performed using the RobusT II RT-PCR Kit (Finzymes, Finland). 50μl of the reaction mixture contained: RNA eluted (5μl), buffer 10X (5μl), MgCl$_2$ 50mM (1.5μl), dNTP mix 10mM (1μl), RNAsi Inhibitor 5U/μl (2μl), M-Mulv 5U/μl (2μl), primers 100ng/μl (1μl) and RNAsi-free water (32.5μl). The RT thermal profile consisted of an initial step of 45 min at 42°C, followed by 2 min at 94°C. The previously synthetisised cDNA was amplified by real-time PCR using the DyNAmo HS SYBR Green qPCR Kit. Each reaction mixture consisted of: cDNA (4μl), Master mix (10μl), primers mix (4μl), ROX-reference dye (0.4μl) and RNAsi-free water (1.6μl). PCR was performed on the DNA Engine Opticon 2 Continuous Fluorescence Detector (MyResearch, Inc-Waltham, Massachussets, USA) by the following means: 15 minutes for the activation of the DyNAmo *hot-start* polymerase at 95°C and simultaneous DNA denaturation, 46 amplification cycles (10 sec at 94°C, 25 sec at 60°C and 30 sec at 72°C), followed by 5 minutes at 72°C for the final extension.

Design of the evaluation
Melting-curve analysis

Influenza A virus sub-typing and the differentiation between the two lineages of influenza B were achieved by melting curve analysis, which had been established between 76°C and 90°C, with a temperature increment of 0.1°C and a hold time of 5 seconds. A total of 90 influenza positive specimens (15 A/H1N1/New Caledonia/20/99-like, 15 A/H3N2/Panama/2007/ 99-like, 16 A/H3N2/Hong Kong/1143/02-like, 22 type B strains belonging to the Victoria/2/87 lineage and 22 strains belonging to the Yamagata/16/88 lineage) were tested and their corresponding melting temperatures (Tm) are presented as box plots. These positive samples were obtained by taking naso-pharingeal swabs

from individuals with Influenza-like-illnesses (ILI) during the seasons of 1999-2004 and confirmed by nested RT-PCR (Influenza/RSV Multiplex, Amplimedical S.p.A, divisione Bioline, Torino).

Specificity

A panel of respiratory viruses commonly found in the respiratory tract was used to determine the specificity of the real–time PCR. Cell culture surnatants, positive for Respiratory Syncytial Virus (RSV), Measles virus, Mumps virus, Adenovirus and Parainfluenza Viruses (PIV) type 1, 2, 3 were tested by real-time PCR. PIV isolates and RSV isolate were subcultured on Hep-2 cells. Measles isolate was inoculated into B95a cells. The viral growth was confirmed by indirect immunofluorescence test. To evaluate the influenza type cross-reactivity, influenza A and B surnatants were processed using influenza B and A primer, respectively. Influenza viruses were cultured onto MDCK and the viral growth was confirmed by the HA test.[10]

Analytical sensitivity

Cell culture-grown A/H1N1, A/H3N2, the Victoria-like and Yamagata-like B viruses were titred using plaque reduction assay and 5 fold serial dilution.[11] Each dilution was processed by six-fold real-time PCR in six different runs.

Clinical sensitivity

Twenty-five respiratory samples, collected from individuals with ILI, during the seasons of 2001/02, 2002/03 and 2003/04 and positive for influenza viruses, confirmed by commercial nested PCR (Influenza/RSV Multiplex, Amplimedical S.p.A, divisione Bioline, Torino) and cell culture, were selected as being representative of the circulating strains. In particular, 7 of the specimens were A/H1N1/New Caledonia/20/99-like, 5 A/H3N2/Panama/2007/99-like, 5 A/H3N2/ Hong Kong/1143/02-like, 5 type B strains belonging to the Victoria/2/87 lineage and 3 strains belonging to the Yamagata/16/88 lineage. The HA phylogenetic tree including A/H3N2 and B isolates used to evaluated the clinical sensitivity are shown in Figure 1. Detection as well as molecular and serological characterisation of the influenza viruses were performed as previously described.[6]

Results
Melting-curve analysis

The Tm value distribution of A/H1N1, A/H3N2, the Victoria-like and the Yamagata-like B viruses are shown in Figure 2. There was a clear

Figure 1. A phylogenetic tree including tested viruses isolated in Italy during the 2001/02, 2002/03 and 2003/04 seasons along with reference strains.

Figure 2. Tm value distribution of A/H1N1, A/H3N2, Victoria-like and Yamagata-like B viruses. Box plot show the median, interquantile range, outliers and extreme cases of variables.

virus showed negative results, without any fluorescence increase. Only the highly concentrated influenza virus B showed a fluorescence increase after 37 cycles, although the observed Tm was out of the range for the A/H1N1 and A/H3N2 viruses and then the sample was considered negative. The sample was re-tested at several dilutions, confirming the previous data. Using primer B, all of the tested viruses showed no fluorescence increase.

Analytical sensitivity

Using real time PCR, a positive result was obtain in every run for the following dilutions: 0.64 PFU for A/H1N1, 0.026 PFU for A/H3N2, 0.64 PFU for type B Victoria-lineage and 0.62 PFU for the B Yamagata-lineage. A positive result was obtained in 1 out of every 6 runs for the A/H1N1 dilutions corresponding to 0.13 PFU.

Clinical sensitivity

Out of the 25 respiratory samples, selected as representative of the circulating strains and with a positive nested-PCR, 22 (88%) were positive. The 3 discordant results, 1 sample subtyped A/H1N1, 1 A/H3N2 and 1 belonging to the Victoria lineage, were re-tested using real-time PCR and nested PCR. Surprisingly, all of the samples were negative for both assays.

Discussion

In this report we describe a new real time PCR assay based on melting curve analysis for the detection, typing and subtyping of influenza viruses, using an approach that had been previously been applied for other viruses, but not yet for orthomyxovirus.[12-15] The Tm value distribution showed a clear discrimination between the different type B influenza lineages, while subtypes of type A can also be differentiated, the temperature ranges were close. The evaluation of specificity showed that, by also testing highly concentrated samples positive for other respiratory viruses, specific templates were not produced and influenza type cross-reactivity was not observed.

As far the analytical and clinical sensitivity is concerned, the new real-time PCR showed that the lower detection limit was constantly lower than 1 PFU for every virus, and the new assay was able to detect every type, subtype and variant circulating during the previous seasons.

The results obtained by the analysis of respiratory specimens with real-time PCR were in accordance with those obtained by nested PCR, except for 3. The reason for this discrepancy is

discrimination between the different viruses: the ranges for the Tm obtained were 79.3-80.1°C, 80.2-81.5°C, 78.2-79.1°C, 80.2-80.6°C for A/H1N1, A/H3N2, the B/Victoria and the B/Yamagata influenza viruses. The Tm distribution of A/H3N2/Panama/2007/99-like and A/H3N2/ Hong Kong/1143/02-like isolates were overlapping (data not shown).

Specificity

Using primer A, surnatants positive for RSV, PIV 1, 2 and 3, adenovirus, mumps virus and measles

probably due to RNA degradation which occurred between the sample collection and the complete fulfilment of the real time assay or contamination during the multiplex PCR assay fulfilment, despite precautions being undertaken.

Other advantages of the new assays are the reduced contamination risk, having no post-amplification manipulations and being less time-consuming, thus it has an important impact on patient care. The time taken to reach a quick diagnosis can reduce the length of hospitalisation, the nosocomial spread and improve patient management by implementing the appropriate therapy, thus helping to decrease the extent of outbreaks.

In conclusion, the new assay provides an accurate and sensitive diagnosis for influenza virus infections and represents a sensitive tool for virological surveillance and the management of patient with ILI.

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