Journal of Clinical Virology 57 (2013) 222–226

Contents lists available at SciVerse ScienceDirect

Journal of Clinical Virology

journal homepage: www.elsevier.com/locate/jcv

Evaluation of a polymerase chain reaction–electrospray ionization time-of-flight mass spectrometry for the detection and subtyping of influenza viruses in respiratory specimens

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ARTICLE INFO

Article history:
Received 14 November 2012
Received in revised form 27 February 2013
Accepted 6 March 2013

Keywords:
Influenza
Mass spectrometry
Respiratory viruses
Multiplex PCR
Direct diagnosis

ABSTRACT

Background: PCR coupled to electrospray ionization mass spectrometry technology (PCR/ESI-TOF-MS) (PLEX-ID system, Abbott Ibis Biosciences) was developed to characterize microbial pathogens.
Objectives: To evaluate the performance of the PLEX-ID flu detection™ kit for detecting Influenza viruses by comparison with the multiplex RespiFinder® Kit (PathoFinder).
Study design: Acute-phase respiratory samples ($n = 293$) were analysed for this purpose. A subpopulation of influenza type A positive samples, identified with the RespiFinder® kit ($n = 64$), were subtyped with the RealTime ready Inf A/H1N1 Detection Set® (Roche Molecular Diagnostics) and results were compared to the PLEX-ID Flu Detection™ kit.
Results: 274 samples gave concordant results (93.5%, $p < 0.0001$): 65 influenza A-positive, 18 influenza B-positive and 191 negative samples. Of these, 7 samples were PLEX-ID positive/RespiFinder® negative (5 influenza A and 2 influenza B) and 12 were PLEX-ID positive/RespiFinder® negative (10 influenza A and 2 influenza B). PLEX-ID showed one sample as an influenza A and B co-infection while the RespiFinder® assay showed it to be influenza A-positive. The sensitivity, specificity, positive and negative predictive values of the PLEX-ID™ system were 87.4%, 96.5%, 92.2% and 94.1% respectively. Thirteen of 19 discordant samples available for retesting were investigated further with the Anyplex™II RV16 Detection kit (Seegene): seven were RespiFinder® concordant, while six were PLEX-ID™ concordant. Subtyping of 61/64 influenza A samples was concordant (95.3%): 55 were H1N1pdm09 and six were non-H1N1pdm09. Three samples gave negative PLEX-ID™ results (one H1N1pdm09 and two non-H1N1pdm09).
Conclusions: PCR/ESI-TOF-MS technology showed good diagnostic performances to detect and subtype influenza viruses.

1. Background

Influenza viruses are responsible for winter epidemics among children and adults, with huge potential costs for society.[1] Influenza viruses are detected directly from respiratory samples.[2] That was originally done using cell cultures or direct antigen detection [3] but culture-based methods are time-consuming and direct antigen detection is not sensitive enough to provide a reliable diagnosis. Molecular diagnostics, which is globally the current standard technology, overcomes these limitations.[4] The majority of molecular approaches to influenza viruses detection are based upon real-time reverse-transcription PCR targeting the segment encoding the conserved matrix protein (*M*) gene segment.[5] Downstream assays can be used to type influenza A or B or further subtype influenza A.

Some influenza A subtypes (H1N1pdm09, non-H1N1pdm09, H3N2, avian H5N1) can be detected using current molecular techniques that target hemagglutinin (*HA*) and neuraminidase (*NA*).[6] However, it is still difficult to detect and identify emerging viruses with reassorted internal genes.

New technologies like the polymerase chain reaction (PCR) electrospray ionization time-of-flight mass spectrometry (PCR/ESI-TOF-MS) should be able to identify these pathogens. The method

Abbreviations: PCR–ESI-TOF-MS, PCR-electro-spray ionization time-of-flight mass spectrometry; RT-PCR, reverse-transcription PCR; MLPA, multiplex ligation-dependent probe amplification; RSV, respiratory syncytial virus.

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http://dx.doi.org/10.1016/j.jcv.2013.03.004

was originally applied in microbiology,[7] for identifying species of bacteria in respiratory samples,[8] but it has also been used to identify viruses including coronavirus [9] and adenovirus.[10] PCR/ESI-TOF-MS technology has more recently been used to identify and characterize the recombinant influenza A H1N1pdm09 [11–14] and to identify the origin of the gene segments of that reassortant virus of porcine, human and avian influenza viruses.[15,16] The technique has also been independently evaluated for its ability to type influenza in retrospective nasopharyngeal specimens.[17] The present study is the first showing results of samples collected prospectively and compared to a multiplex reference method different from any previously published method.

2. Objectives

This study evaluates the suitability of the PLEX-ID flu detection™ assay based upon a reverse-transcription (RT) coupled to PCR/ESI-TOF-MS, for detecting influenza viruses in prospective clinical respiratory samples and determining their subtypes.

Samples tested with the multiplex RespiFinder kit® [18,19] were used as a reference for comparison and discrepant results were tested with a third RT-PCR multiplex molecular technique for confirmation of results.

We discriminated between H1N1pdm09 and non-H1N1pdm09 influenza A subtypes in a subpopulation of samples that were influenza A positive with the RespiFinder kit® using the RealTime ready Inf A/H1N1 Detection Set®. The results were compared to those obtained with the PLEX-ID Flu detection® assay.

3. Study design

3.1. Materials

The Department of Virology, CHU Toulouse, France, tested 293 respiratory samples prospectively collected during the 2010–2011 winter season when the Flu epidemic was on its peak. Of these, 148 were from the French Flu survey network and 145 were collected from children attending the Pediatric Unit of the Toulouse University Hospital.

Nasopharyngeal swab samples were collected on Virocult® swabs (Kitvia, Labarthe Inard, France) and stored at $-80 \pm 5\,^{\circ}C$.

3.2. Methods

3.2.1. Multiplex ligation-dependent probe amplification (MLPA)

Respiratory samples were tested prospectively with the MLPA. Total nucleic acid was extracted with the MagNA Pure LC Total Nucleic Kit® (Roche) on the MagNA Pure LC™ instrument according to the manufacturer's instructions. Input sample volume was 200 μl and output elution volume was 100 μl (2-fold concentration).

Respiratory viruses were detected with the RespiFinder® DC kit, which can detect 15 respiratory viruses: influenza virus type A, influenza virus type B, avian influenza virus A/H5N1, respiratory syncytial viruses (RSV) A and B, parainfluenza viruses 1, 2, 3 and 4, coronaviruses OC43, 229E and NL63, rhinovirus, adenovirus and human metapneumovirus.

The method is based on amplifying viral genomes with a multiplex RT-PCR. Its sensitivity is due to hybridization, ligation and PCR of the probe. The various amplicons are detected by size-fractionation on a capillary electrophoresis system with the sequencing analyzer ABI 3130 XLS (Applied Biosystems). The internal amplification control is an RNA transcript from the encephalomyocarditis (EMC) virus: this checks for the presence of sample addition and the absence of PCR inhibitors.

3.2.2. Influenza A virus subtyping using real time PCR

The MLPA test does not differentiate between influenza A H1N1pdm09 and influenza A non-H1N1pdm09 viruses. The influenza type A viruses detected were subtyped by testing influenza A positive MagNA pure total nucleic acid extracts with the RealTime ready Inf A/H1N1 Detection Set® (Roche Diagnostics) employing the RNA virus master® one-step RT-PCR kit (Roche Diagnostics) according to manufacturer's instructions on the Light Cycler 480™ system (Roche Diagnostics, Meylan, France). The set contained specific primer/probe mixes for detecting influenza A matrix protein 2 (*M2*) and the H1N1pdm09-specific hemagglutinin HA1 (*H1*) in a single reaction.

3.2.3. PCR–electrospray ionization time-of-flight mass spectrometry (PCR/ESI-TOF MS)

Respiratory samples were analyzed with the PLEX-ID system (Abbott Molecular, IL, USA). RNA was extracted from nasopharyngeal swabs using the PLEX-ID Viral RNA Isolation Kit (Abbott Molecular, IL, USA) on the PLEX-ID FH and SP instruments in a fully automated manner. Input sample volume was 300 μl and output elution volume was 200 μl (1.5-fold concentration).

The PLEX-ID Fluid handler was used to distribute the nucleic acids and enzymes for PCR into the PLEX-ID Flu Detection plates. Viral nucleic acids were amplified using the PLEX-ID Flu Detection™ kit (Abbott Molecular, IL, USA). Segments of the influenza genome were amplified by PCR on the PLEX-ID TC (Mastercycler ProS–Eppendorf) using nine primer-pairs located at core conserved sites.

The regions targets are shown in Table 1. The Pan-influenza-PB1 primer-pair detects all influenza viruses (A, B and C types). Primer-pairs targeting nucleoprotein (*NP*), matrix protein (*M1*), polymerase (*PA*), non-structural 1 (*NS1*) and polymerase basic 2 protein (*PB2*) gene segments were designed to amplify sequences of influenza A viruses only. Primer-pairs located on *HA* and *NA* glycoproteins were designed to amplify only influenza H1N1pdm09. A specific PB2 primer was selected to amplify influenza B in addition to the pan-influenza primer. The last well contained the PB2 and the influenza A-*NA* (N1-TFR) which covers the most common mutation (N1-TFR) associated with oseltamivir (Tamiflu) resistance (H275Y).[20,21]

Each amplicon is automatically desalted and purified on a weak anion exchanger.[22] Methanol-based aerosols containing denatured ionized amplicons are sprayed into the mass spectrometer. The time-of-flight to the detector is determined giving the mass of the amplicon.[12] By database analysis, the base composition (BC) of each PCR amplicon derived from each viral gene segment is determined. Influenza viruses are then typed and subtyped by bioinformatic analysis of the BC signatures produced by the nine target genes. The BC signatures are compared to known BC signatures in the reference database (Influenza Research Database, NCBI Influenza Virus Resource, Systems Influenza.org.) and viruses are identified by matching them to the closest signatures in the database.

3.2.4. Analysis of discordant results

The Anyplex™II RV16 Detection kit (Seegene) was used as a third analytical technique for samples that gave discordant results. This multiplex RT-PCR test detects 16 viruses, including influenza A and B.

3.2.5. Statistical methods

Data were analyzed using StatView 5.0 StataTM software (StataCorp, Texas). The Kappa-Cohen test was used to compare assays. *P* values less than 0.05 were considered significant. The sensitivities (percentages of reactive samples) of these assays were compared using the McNemar's chi-squared test.