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Short communication

Semiquantitative one-step RT-PCR for simultaneous identification of human influenza and respiratory syncytial viruses

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Abstract

A multiplex reverse transcription-polymerase chain reaction (mRT-PCR) method was developed for simultaneous detection and typing/subtyping of influenza viruses A/H1, A/H3 or B, and respiratory syncytial viruses A or B, followed by DNA semiquantitation using the Agilent 2100 Bioanalyzer. Such method provides a rapid, specific and sensitive diagnostic tool for detection and semiquantification of respiratory illness specimens.

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Acute respiratory tract infections (ARIs) are the most common causes of morbidity and mortality in young children, the elderly and immune-compromised patients worldwide (Boivin et al., 2004; Hinman, 1998). Among the major viral causative agents of ARI are influenza A and B viruses and the respiratory syncytial viruses (RSVs) A and B (Stockton et al., 1998). Rapid diagnosis of these viral pathogens is important for determining appropriate clinical intervention and for monitoring these or related viruses during outbreaks or pandemic of emerging pathogens.

A number of techniques are available for detection and characterization of influenza viruses and RSV including conventional methods such as cell culture, enzyme immunoassay (EIA) and immunofluorescence (IF). Recent advances in molecular techniques enable detection of pathogens based on the nucleic acid sequences of genetic material isolated from viruses or bacteria in clinical specimens. Notably, polymerase chain reaction (PCR)-based molecular methods have been used widely and are well accepted for laboratory diagnosis of pathogens due to their rapidity, sensitivity and high throughput capability (Fredricks and Relman, 1999; Louie et al., 2000; Weinberg et al., 2002; Yang and Rothman, 2004). In the past few years, several multiplex reverse transcription-polymerase chain reaction (mRT-PCR) methods have been developed for the detection of influenza viruses, RSV and parainfluenza viruses (PIVs), etc. (Bellau-Pujol et al., 2005; Coiras et al., 2003; Grondahl et al., 1999; Stockton et al., 1998; Syrmis et al., 2004). In these methods, analysis of PCR products relies either on agarose gel electrophoresis or more time-consuming enzyme-linked assays and many of them include a nested PCR step to enhance sensitivity. However, none of the published methods provide quantitative results. More recently, real-time RT-PCR assays for detecting influenza and RSV have been developed and used successfully in testing clinical specimens (Boivin et al., 2004).

Here, we report on the development of a rapid, specific, and sensitive mRT-PCR method for simultaneous detection of influenza A/H1, A/H3 or B and RSV A/B. The primers for influenza A viruses were selected from the nucleotide sequences encoding the hemagglutinin (HA) gene as follows: for A/H1 subtype, 5'-atgcagacacaatatgtataggc-3' (HA1-forward [F]) and 5'-gatactgagctcaattgctc-3' (HA1-reverse [R]); for A/H3 subtype, 5'-gagctggttcagagttcctc-3' (HA3-F) and 5'-gtgacctaagggaggcatataatc-3' (HA3-R). The primers for influenza B viruses were selected from the nucleotide sequences encoding the nucleoprotein (NP) gene as follows: for A/H1 subtype, 5'-gtcagacacaaatgtataggc-3' (HA1-forward [F]) and 5'-gtcagacacaaatgtataggc-3' (HA1-reverse [R]); for A/H3 subtype, 5'-gagctggctgctatgtataggc-3' (HA3-F) and 5'-gtacactaataggcataaatc-3' (HA3-R). The primers for influenza B viruses were selected from the nucleotide sequences encoding the nucleoprotein (NP) gene as follows: 5'-gaagtaggtggagacggaggg-3' (NP-F) and 5'-gtacactaataggcataaatc-3' (NP-R). The primers for RSV were selected from the nucleotide sequences encoding the nucleocapsid protein (NP) gene

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Fig. 1. Analysis of mRT-PCR products by DNA 500 LabChip. (Lanes 1–9) viral RNA was diluted and mixed at 1:1 ratio for A/H1, A/H3, B or RSV A/B as described in individual lane. Dilutions of RNA for A/H1, A/H3 and B are 10,000 fold; for RSV A2, 1000 fold; for RSV 2B, 10,000 fold. Lane 1, A/H1 + A/H3 + B + RSV(A + B); lane 2, B + RSV(A + B); lane 3, A/H1 + RSV(A + B); lane 4, A/H3 + RSV(A + B); lane 5, A/H1 + B; lane 6, A/H3 + B; lane 7, A/H1 + A/H3 + B; lane 8, A/H1 + B + RSV(A + B); lane 9, A/H3 + B + RSV(A + B). The sizes of the PCR fragments are 330 bp (A/H1), 210 bp (A/H3), 390 bp (B), 133 bp (RSV A/B), respectively.

as follows: for RSV-A, 5′-cagaggtacccgagaatcaa-3′ (RSVANP-F) and 5′-gctgatatgtttagctct-3′ (RSVANP-R); for RSV-B, 5′-tagaggtacccgagaatcaa-3′ (RSVBNP-F) and 5′-gctgatatgtttagctct-3′ (RSVBNP-R). The PCR assay was evaluated by testing primer pairs against various laboratory virus stocks. Briefly, viral RNA was extracted from 70 µl influenza and RSV virus stocks or respiratory specimens using the QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA). Viral particle counts were determined by electron microscopy (Advanced Biotechnologies Inc., Columbia, MD) as previously described (Hu et al., 2003). Multiplex RT-PCR was carried out in a 9600 thermal cycler (Applied Biosystems Inc., Foster City, CA) using the Titan One Tube RT-PCR kit (Roche, Indianapolis, IN) in a total volume of 50 µl with 5 µl RNA and 2 µl master primer mix. The master primer mix comprised 75 µl A/H1, 50 µl A/H3, 60 µl B, 50 µl RSV-A and 50 µl RSV-B primers (including both forward and reverse sequences). All stock primer concentrations were 160 ng/ml. The PCR program was 48 °C for 15 min, 52 °C for 30 min and 94 °C for 2 min followed by 40 cycles of 94 °C for 1 min, 60 °C for 30 s and 68 °C for 1 min. Final extension was carried out at 68 °C for 5 min. The amplification products were analyzed by DNA 500 LabChip using the Agilent 2100 bioanalyzer according to the manufacturer’s instructions (Agilent Technologies, Palo Alto, CA). Briefly, 1 µl PCR reaction was loaded into one of 12 sample wells on a LabChip containing 5 µl DNA 500 Markers and run for 30 min under appropriate settings.

To evaluate specificity and sensitivity, the following virus stocks with known particle numbers were used as positive controls: A/H1N1/New Caledonia, 3.6 × 10⁹/ml; A/H3N2/Panama, 2.1 × 10⁹/ml; B/Johannesburg, 1.8 × 10⁹/ml; RSV A2, 7.6 × 10⁸/ml; RSV 2B, 1.02 × 10⁹/ml. Fig. 1 shows the DNA 500 LabChip analysis of the mRT-PCR products with various combinations of the viruses. Non-specific PCR products were not observed. In addition, the sensitivity of the RT-PCR was evaluated by performing PCR in duplicate for each type of viral RNA independently. For these stocks, the minimal number of viral particles detected in the assay were equivalent to: A/H1N1, 2.1 × 10³; A/H3N2, 1.2 × 10³; B, 1.1 × 10³; RSV A, 4.4 × 10; RSV B, 6.0 × 10³, respectively (data not shown).

This mRT-PCR assay was utilized to evaluate 56 nasal swab specimens obtained from Asian children (6–36 months old) with respiratory symptoms, who were enrolled in an experimental influenza vaccine trial conducted in Malaysia, Singapore, Thailand and India during the winter of 2000 and 2001. Among these specimens, the mRT-PCR detected 10 influenza A/H1, 6 influenza A/H3, 8 influenza B positive and 1 RSV (A/B) positive sample (Table 1), whereas culture confirmation identified 14 influenza A and 7 influenza B positive samples. No RSV culture was performed for these specimens. The PCR detected all culture-positive specimens with the additional benefit of distinguishing A/H1 and A/H3 subtypes. In addition, three culture-negative samples tested positive by the mRT-PCR method, one each from influenza A/H1, A/H3 and B groups, respectively.
Despite the relatively small sample size, we further validated the results using a real-time RT-PCR assay performed on the same specimens. The results confirmed all the PCR positives and showed that compared with the culture-positive specimens, the three culture-negative specimens that were positive by PCR contained relatively low copy numbers of the viruses, ranging from 22 to 721 copies per reaction (data not shown).

Respiratory viruses are conventionally diagnosed based on cell culture or antigen detection such as EIA and IF, which can be time-consuming or have limitations in sensitivity and specificity. Although real-time PCR may have higher sensitivity than conventional PCR, the latter is usually more economical for clinical laboratories that are not equipped with expensive real-time PCR instruments. In addition, the optimal of multiplicity for real-time PCR is currently limited to three due to a limited number of fluorophores (S. Cheng, unpublished). The method reported in this study, which primarily targets influenza A/H3, A/H1 and B viruses and RSV A and B, is carried out in a single-step reaction with minimal opportunity for contamination since no secondary or tertiary nested PCR is needed as in previous reports in this study, which primarily targets influenza A/H3, A/H1 and B viruses and RSV A and B, is carried out in a single-step reaction with minimal opportunity for contamination since no secondary or tertiary nested PCR is needed as in previous methods (Bellau-Pujol et al., 2005; Coiras et al., 2003; Grondahl et al., 1999; Stockton et al., 1998). The PCR amplicons in this method range from 133 to 390 bp, which are relatively small in size that can increase the assay sensitivities. For analyzing PCR products, the DNA LabChip platform of Agilent Technologies was utilized to replace conventional agarose gel electrophoresis or more time-consuming enzyme-linked hybridization assays. The LabChip assay not only consumes smaller sample volumes (1 µl per reaction) but also enhances sensitivity to as low as 0.5 ng/µl DNA. The LabChip provides unambiguous, consistent and semi-quantitative results, which permits ease of quality control and quantity estimation for any downstream applications, such as DNA sequencing for strain determination. It also provides information such as relative abundance of viruses in the case of mixed infections. Our results showed that the mRT-PCR assay could easily detect specimens containing low virus copy numbers as well as cases of virus co-infection (e.g. 2 cases of A/H1 and A/H3 and 2 instances of A/H1 and B), which were not detected by culture confirmation. One of the limitation of this method is that the size of the PCR products for RSV A and B are not distinguishable; however, a separate assay designed to address this limitation was developed later (Hu et al., 2003).

In conclusion, our mRT-PCR provides a rapid, sensitive and semi-quantitative tool for the detection and typing/subtyping of influenza viruses and RSV, and can be applied to screen respiratory illness specimens obtained from patients with similar clinical respiratory symptoms. The assay can be easily performed by local laboratories for determination of the causative viral agent(s) of respiratory illness during suspected outbreaks of newly emerging pathogens, such as severe acute respiratory syndrome (SARS) virus, and potential avian flu H5N1 virus, by eliminating common viral pathogens, thus facilitating local disease management and global surveillance.

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