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Evaluation of the Cepheid Respiratory Syncytial Virus and Influenza Virus A/B real-time PCR analyte specific reagent

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A B S T R A C T

Two rapid real-time RT-PCR assays, specific for respiratory syncytial virus (RSV) and influenza A and B, were evaluated for the detection of these viruses in clinical respiratory samples. The RSV assay was applied to 100 samples and the Influenza A and B assay applied to 96 samples all of which had been tested previously by an "in-house" multiplex real-time PCR assay. Forty-three samples were negative for RSV by both methods and 56 samples were positive by both methods. One sample was negative by the new RSV assay although it was positive for RSV A by the "in-house" test. Thirty-nine samples were negative for influenza virus by both methods and 55 samples were positive by both assays. Two samples were negative by the new influenza assay however they were positive by the "in-house" influenza assay. The new assays did not cross react with samples containing other viruses including parainfluenza 1, 2, and 4; human metapnuemovirus; coronavirus 229E, NL63, OC43; rhinovirus; adenovirus; bocavirus and had a specificity of 100% and a sensitivity of 98.2% for RSV and 96.5% for influenza respectively. The results of this study demonstrate that the new assays are specific and sensitive for the detection of RSV and influenza viruses in clinical samples.

1. Introduction

Acute respiratory tract infections are a significant cause of morbidity and mortality especially during the winter months. The elderly, the immunocompromised and patients with respiratory problems such as emphysema and asthma are particularly vulnerable to developing severe disease. Viruses that commonly cause respiratory illness include influenza virus, parainfluenza virus, respiratory syncytial virus (RSV), adenovirus, and human metapneumovirus. These viruses produce a range of symptoms including fever, rhinitis, pharyngitis and myalgia to more serious complications such as bronchitis, bronchiolitis, pneumonia and death (Boivin et al., 2004). Influenza virus and RSV are among the most common viral agents and rapid identification of the aetiology of infection allows better patient management in terms of treatment and infection control (Woo et al., 1997). Rapid detection of viral respiratory pathogens is also important for monitoring emerging influenza strains which may cause epidemics or pandemics. Conventional detection methods for influenza virus include cell culture, antigen detection, serological tests, and immunofluorescent antibody-based (IFA) methods. However, these tests are either too slow to allow timely diagnosis or they lack sensitivity and specificity. More recently immuno-chromatographic point of care tests (POCT) have been developed which have fast turn around times (Aslanzadeh et al., 2008; Selvarangan et al., 2008) however these assays often have limitations in sensitivity and specificity when compared to molecular tests (Boivin et al., 2004). The development of molecular diagnostic assays with superior sensitivity and significantly reduced turn around times when compared to conventional methodologies has led to them being considered to be the "gold standard" for respiratory virus detection (Leven, 2007). Numerous real-time RT-PCR assays for the detection of influenza virus and RSV have been described in either monoplex (Gueudin et al., 2003; Kuypers et al., 2004; Smith et al., 2003; Stone et al., 2004; van Elden et al., 2001) or multiplex format (Brittain-Long et al., 2008; Gunson et al., 2005; Hymas and Hillyard, 2009). The Regional Health Protection Agency Newcastle Laboratory tests all respiratory samples with a panel of "in-house" multiplex real-time PCR assays which target influenza A and B, parainfluenza 1–4, RSV A and B, rhinovirus, adenovirus, human metapnuemovirus; and coronaviruses NL63, OC43, 229E. However it is not feasible to provide urgent out of hours testing based on this multiplex format therefore alternative virus specific testing is preferable for urgent individual samples. A rapid, real-time PCR-based diagnostic test for RSV and influenza A and B which can be performed in a relatively short turn around time would be extremely useful for patient management (Goodrich and Miller, 2007). In addition, the resulting improved treatment of patients presenting with respiratory illness would help infection

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control, reduce nosocomial spread, and reduce the length of patient hospital stay (Woo et al., 1997).

The SmartCyler is a rapid random access, modular real-time PCR instrument which can perform 40 PCR cycles in less than 1 h (Habib-Bein et al., 2003). The aim of this study was to evaluate two multiplex real-time RT-PCR assays on the rapid SmartCycler real-time PCR system for the detection of influenza A and B and RSV. The assays used the Respiratory Syncytial Virus Analyte Specific Reagent (ASR) bead and the Influenza A/B ASR bead (Cepheid, Sunnyvale, USA) which contain PCR primers and probes in a lyophilized bead format. The RSV ASR bead contains primers and FAM-labelled probes targeting the N (nucleocapsid protein) gene of RSV types A and B. The Influenza A/B ASR bead contains primers and a FAM-labelled probe and an Alexa Fluor 532-labelled probe for the detection and differentiation of influenza A and B, respectively. When reconstituted the ASR bead provides sufficient primers and probes for two 25 μl PCR reactions. The influenza A and B ASR reagents have not been evaluated previously to determine their utility for the detection of influenza in clinical samples from patients with respiratory disease. In this study, the ASR reagents were evaluated in real-time RT-PCR assays using the SmartCycler real-time PCR instrument. The new assays were applied to the detection of influenza virus and RSV in a collection of samples previously tested using an "in-house" multiplex real-time RT-PCR assay.

2. Materials and methods

2.1. Samples and nucleic acid extraction

A collection of 196 respiratory samples from patients with acute respiratory tract infections were tested in this study. Samples were collected previously during the 2004–2008 winter respiratory seasons. Sample types included nasopharangeal aspirates, nasal swabs, throat swabs, sputum, tracheal secretions, and bronchoalveolar lavages. Of the 196 samples, 57 had been shown previously to be positive for RSV (38 Type A, 18 Type B, one dual infection A and B) and 48 samples positive for influenza virus (31 influenza A and 17 influenza B) by two multiplex real-time RT-PCR assays targeting the nucleocapsid protein gene (N gene) of RSV as described previously (Gunson et al., 2005), and the influenza A matrix gene and influenza B N gene as described previously (Anon, 2006; Curran et al., 2007). These "in-house" assays were performed using a TaqMan FAST 7500 real-time PCR instrument. The majority of the samples had also been tested by IFA and all IFA negative samples were tested by cell culture. Prior to nucleic acid extraction all samples were stored at −80 °C. Total nucleic acid was extracted using the Roche MagNa Pure instrument and Roche MagNa Pure Total NA Large Volume Kit (Roche Diagnostics, Lewes, UK) according to the manufacturer's protocol. Two hundred microlitres of each sample were extracted and the nucleic acid extract eluted in 60 μl of elution buffer (Roche Diagnostics, Lewes, UK). Prior to PCR testing nucleic acid extracts were stored at −80 °C.

2.2. Real-time RT-PCR using the Respiratory Syncytial Virus and Influenza Virus A/B real-time PCR analyte specific reagent

Viral RNA was reverse transcribed and the resulting cDNA amplified by PCR using the SuperScript III Platinum One-step Quantitative RT-PCR kit (Invitrogen, Paisley, UK) in a total reaction volume of 25 μl. The RSV and influenza ASR beads were reconstituted in 14 μl PCR-grade water, 25 μl of SuperScript III Platinum One-step Quantitative RT-PCR 2× reagent (containing a proprietary buffer system, $MgSO_4$, dNTP's and stabilizers), and 1 μl of the SuperScript III reverse transcriptase/Platinum *Taq* mix. Twenty microlitres of this master mix was then aliquoted into two individual SmartCycler reaction tubes, one per test and 5 μl of template nucleic acid added. The final RSV reaction mix contained: 3 mM $MgSO_4$, 0.2 μM RSV A forward primer; 0.4 μM RSV A reverse primer; 0.2 μM RSV B forward primer; 0.4 μM RSV B reverse primer; 0.1 μM RSV A FAM-labelled probe; 0.15 RSV B FAM-labelled probe; 4.375 mM HEPES (pH 8.1); 0.5 μl of the SuperScript III reverse transcriptase/Platinum *Taq* mix. The final Influenza A/B reaction mix contained: 3 mM $MgSO_4$, 0.6 μM influenza A forward and reverse primers; 0.3 μM influenza B forward and reverse primers; 0.1 μM influenza A FAM-labelled probe; 0.15 influenza B Alexa Fluor 532-labelled probe; 4.375 mM HEPES (pH 8.1); 0.5 μl of the SuperScript III reverse transcriptase/Platinum *Taq* mix. Amplification was carried out in a SmartCycler Real-time PCR system (Cepheid, Sunnyvale, USA) with thermal cycling conditions for the influenza A/B assay being 50 °C for 30 min, 95 °C for 15 min, and 40 cycles of 94° for 15 s, 60 °C for 45 s, and 72 °C for 15 s. Thermal cycling conditions for the RSV A/B assay were 48 °C for 15 min, 95 °C for 15 min, and 50 cycles of 95° for 15 s, 60 °C for 30 s, and 72 °C for 15 s.

3. Results

3.1. Real-time PCR assay correlation for the detection of respiratory syncytial virus

The SmartCycler RSV assay was applied to 100 samples tested previously by the multiplex "in-house" assay. Forty-three samples were negative for RSV by both methods with 56 samples being positive by both assays. One sputum sample was negative by the SmartCycler RSV assay although it was positive for RSV A by the "in-house" real-time PCR test. This sample was re-tested by the SmartCycler assay however it remained negative for RSV using the new test. Of the 56 samples positive for RSV, 13 also contained other co-infecting viruses including rhinovirus ($n = 8$), bocavirus ($n = 2$), human metapneumovirus ($n = 2$), adenovirus ($n = 1$) and coronavirus 229E ($n = 1$). Of the 43 samples negative for RSV in the SmartCycler assay, 27 were shown to be positive for other viruses including rhinovirus ($n = 14$), human metapnuemovirus ($n = 5$), bocavirus ($n = 3$), parainfluenza virus 1 ($n = 1$), parainfluenza virus 2 ($n = 1$), parainfluenza virus 4 ($n = 3$). Amplification of nucleic acids from any of these 27 samples produced no cross-reactions with the SmartCycler RSV assay. Overall, when compared to the "in-house" multiplex assay the Smartcycler RSV assay demonstrated a specificity of 100% and a sensitivity of 98.2%.

3.2. Real-time PCR assay correlation for the detection of influenza virus

Of the 96 samples investigated, 39 were negative and 55 were positive for influenza virus by both the SmartCycler and "in-house" multiplex PCR methods. The 55 positive samples included 37 positive for influenza A and 18 positive for influenza B. One sample was positive for both influenza A and B using the "in-house" multiplex assay however only influenza A was detected using the SmartCycler assay. Two nasal/throat swab samples were positive for influenza A by the "in-house" PCR but were negative by the SmartCycler influenza assay and on re-testing these samples remained negative. Of the 39 samples negative by the SmartCycler influenza assay 19 were shown to be positive for other viruses including rhinovirus ($n = 6$), human metapnuemovirus ($n = 2$), bocavirus ($n = 2$), parainfluenza virus 1 ($n = 2$), RSV ($n = 2$), adenovirus ($n = 2$), coronavirus OC43 ($n = 2$), and coronavirus 229E ($n = 1$). Amplification of nucleic acids from any of these 19 samples produced no cross-reactions with the SmartCycler RSV assay. Overall, when compared to the "in-house" multiplex assay the Smartcycler influenza assay demonstrated a specificity of 100% and a sensitivity of 96.5%.

4. Discussion

The development of multiplex real-time PCR-based methods for the detection of respiratory viruses has significantly improved the diagnosis of respiratory disease (Leven, 2007). Multiplex assays targeting a wide range of individual viruses are now considered to be the gold standard for diagnosis of viral respiratory disease and test results can be achieved within a few hours of samples being received in the laboratory (Stone et al., 2004). However the majority of laboratories batch samples together and perform one or more test runs per day therefore turn around times can be slower than other rapid tests such as those used as POCT. In addition it is not feasible for many laboratories to provide multiplex PCR testing outside of the normal laboratory opening hours therefore alternative PCR-based methods which can be performed using a random access real-time PCR instrument would shorten turn around times with obvious benefits for patient care.

In this study two rapid real-time PCR assays were evaluated for the detection of influenza and RSV in respiratory samples using the SmartCycler real-time PCR instrument. The RSV and influenza assays were applied to a total of 196 samples tested previously in our "in-house" multiplex real-time PCR test and comparison of the results demonstrated a concordance between the two tests of 98.5%. The SmartCycler RSV test failed to detect RSV in a single sample which was detected by the "in-house" multiplex assay. This false negative result may have been due to degradation of the sample because the extracted nucleic acids were stored at −80 °C for greater than 1 month following testing by the "in-house" multiplex assay prior to re-testing by the SmartCycler assay. The sample also produced a high *Ct* value (38.2) in the original "in-house" assay indicating it contained relatively low numbers of target virus. The SmartCycler influenza assay failed to detect influenza A in two samples which were positive for influenza A in the "in-house" multiplex assay. Again the extracted nucleic acid from these samples had been stored at −80 °C for greater than 1 month therefore sample degradation may have occurred. One of these samples also had a relatively high *Ct* value (40.5) in the "in-house" assay indicating that it contained low levels of target virus. These three samples were re-tested in the "in-house" assays and the original results confirmed. The SmartCycler assay also failed to detect influenza B in a sample demonstrated to contain both influenza A and B. Again this may have been due to sample degradation during storage or alternatively this may reflect differences in the sensitivity of the SmartCycler assay for the different influenza types. Finally these false negative results in both the SmartCycler RSV and influenza assays may be due to sequence variation in the target gene of the assays which may adversely affect the binding of the primers or probes.

Interestingly the *Ct* values generated by the SmartCycler assays for the positive samples were lower than those of the "in-house" test (e.g. influenza B mean reduction in *Ct* value was 7.4, SD = 1.6) indicating that the SmartCycler assay may be more sensitive than the "in-house" test. Alternatively these differences may be due to the way the two real-time PCR instruments calculated their *Ct* values relative to the automatically calculated threshold and cut offs. The positive predictive value of the SmartCycler tests were 100% for both RSV and influenza and the negative predictive value was 97.7% and 95.1% respectively for RSV and influenza.

The SmartCycler ASR bead-based primers and probes simplified the assay preparation steps, reducing the time taken for assay set up considerably when compared with our "in-house" assay. If this assay plus the random access real-time PCR system was used in conjunction with a rapid nucleic acid extraction instrument such as the Qiagen EZ1 instrument (Barkham, 2006; Dundas et al., 2008) sample turn around times could be significantly reduced and PCR assay set up simplified. This may facilitate the more urgent testing of respiratory samples by real-time PCR methods outside of the normal working day or outside of the batched runs during normal working day. Overall the ASR bead-based assay and the SmartCycler instrument provide sensitive, specific and rapid detection of RSV and influenza virus in clinical samples from patients with respiratory disease. Introduction of rapid real-time PCR methods for the diagnosis of illness caused by RSV and influenza, particularly outside of the normal laboratory working day would improve patient management and treatment.

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