# Application of RT-Bst to enhance detection of pathogenic viruses of the respiratory tract

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**ABSTRACT**

Inefficiency of RT-PCR can be associated with the suboptimal process of reverse transcription as only 40-80% of RNA is converted to cDNA. We employed a novel method, RT-Bst, to enrich the concentration of cDNA for subsequent multiplex PCR detection of selected RNA viruses. The RT-Bst method amplifies cDNA through reverse transcription of viral RNA using reverse transcriptase and amplification of cDNA using *Bst* DNA polymerase. Viral RNA was extracted from 25 nasopharyngeal samples for detection of influenza A, B and C; parainfluenza 1–4; human coronaviruses 229E and OC43; respiratory syncytial virus (RSV) and rhinovirus. Both multiplex one-step RT-PCR and RT-Bst PCR were used to compare their performances for detection of virus sequences. These findings were compared with routine laboratory detection. When using RT-Bst PCR, 28% of samples yielded a viral pathogen compared to 20% with RT-PCR and 12% using routine diagnostic tests. RT-Bst PCR was shown to have particular utility in the detection of RSV RNA as this was present in 20% of the samples studied compared to 8% when using RT-PCR. For one patient, RT-Bst PCR was able to detect RSV five days earlier than conventional hospital diagnostic testing. RT-Bst and RT-Bst PCR can be used as alternative approaches to reverse transcription and one-step RT-PCR, respectively, for sequence-independent amplification of RNA virus sequences and a larger scale analysis of this new diagnostic approach is warranted.

KEY WORDS: Multiplex polymerase chain reaction.
Reverse transcription.
RT-Bst amplification.
Virus detection.

## Introduction

The detection of pathogenic viruses of the respiratory tract has been improved with the development and applications of molecular diagnostic methods.[1,2] PCR and reverse transcription PCR (RT-PCR) are now used frequently for the detection of pathogenic viruses of the respiratory tract.[3–5] The detection of a diverse range of pathogenic microorganisms associated with respiratory tract infections has improved our knowledge of their clinical significance.[6] Cold and flu-like illness (CFLIs) is associated with a wide range of possible pathogenic viruses thus requiring the use of a panel of PCR primers to determine the causative agent of CFLIs.[7] Rhinoviruses are the most common cause (50-80%) of CFLIs.[8,9] Other common pathogenic viruses responsible for an estimated 8-15% of CFLIs include influenza viruses A, B and C, parainfluenza viruses 1–4, coronaviruses 229E and OC43, respiratory syncytial viruses, adenoviruses and enteroviruses.[7,10–12] The detection of all possible pathogenic viruses associated with respiratory tract infections is time- consuming and too expensive for most clinical laboratories. However, a multiplex approach to the detection of the most prevalent pathogenic viruses will go some way towards identifying the cause of clinically important virus-associated respiratory tract infections.

A number of commercially available multiplex PCR-based technologies have been developed such as, a micro-bead suspension array,[13] ResPlex technology,[14] the Infinity system[15] and the Jaguar system.[16] In addition, the Luminex x-TAG system has recently been approved by the US Food and Drug Administration for clinical diagnosis of respiratory tract viruses.[17] These technologies require sophisticated instruments and therefore may not be appropriate or cost-effective in all diagnostic laboratories. The development of affordable, highly sensitive multiplex technology could benefit the diagnosis of viral infections in many laboratories.

One-step RT-PCR and two-step RT-PCR can be used for detection of genomic RNA of viruses.[18,19] In one-step RT-PCR, RNA is added directly to the RT-PCR reaction with sequence-specific primers for simultaneous reverse transcription and PCR amplification. This approach requires relatively greater amounts of RNA than two-step RT-PCR. In two-step RT-PCR, cDNA is prepared separately and added to a PCR reaction which leads to the dilution of the template cDNA and reduces the sensitivity of detection. Therefore, one-step RT-PCR is more often used in diagnostic tests to generate and amplify cDNA in a single reaction tube. However, when the quantity of viral genomes is very low (femtogram level or ~10 copies mixed with large excesses of non-target nucleic acids) the template may not be amplified by PCR due to the Monte Carlo Effect.[20,21] This is an inherent limitation when amplifying templates present at very low levels leading to false-negative results, a risk to the patient and possible onward transmission of the virus. Therefore, there is a clear need for a means by which a low copy number template can be amplified to a level which is detectable using existing diagnostic approaches.

In this study, RT-Bst was used to enrich the concentration

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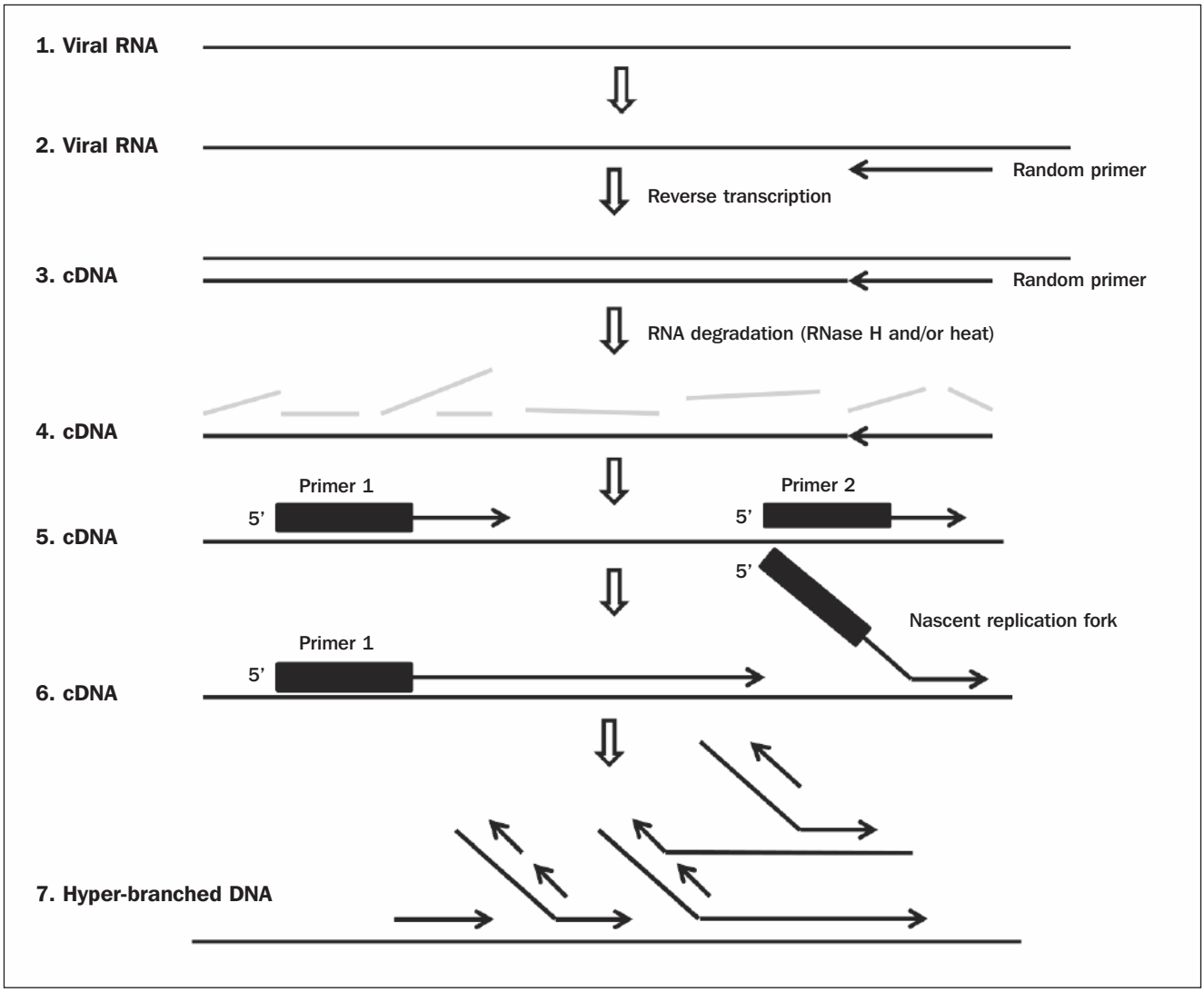


**Fig. 1.** Flow chart of RT-Bst amplification: **1)** viral RNA; **2)** random primers bound to viral RNA; **3)** synthesis of cDNA; **4)** RNA degraded due to RNase H activity or heat; **5)** random primers bound to single-stranded cDNA; **6)** extension of random primers; **7)** synthesis of many copies of single-stranded cDNA (hyper-branched).

of cDNA using Bst DNA polymerase.[22] The hypothetical mechanisms involved for simultaneous reverse transcription and amplification of cDNA is shown in Figure 1. The RT-Bst reaction is unique in that it combines reverse transcription and *Bst* DNA polymerase amplification in a single tube reaction. *Bst* DNA polymerase amplifies the template cDNA using random primers and nucleotides through multiple displacement activity. Random primers bind to different areas of the denatured template and the polymerase extends the primers to synthesise the complementary strand. Random primers proceed and displace the 5′-end of the upstream strands. This reaction is repeated for the displaced strands producing a hyper-branched amplification of cDNA.

*Bst* DNA polymerase has been used for the amplification and detection of DNA and RNA sequences using loop-mediated isothermal amplification (LAMP) and reverse transcription loop-mediated isothermal amplification (RT-LAMP).[23–25] These are alternative approaches to PCR for isothermal amplification of a small fragment (~200 bp) of DNA. However, using these approaches it is not possible to amplify the entire cDNA molecule which can be achieved when using RT-Bst.

In the case of respiratory tract infection it is often necessary to detect a panel of pathogenic viruses, the majority of which are RNA viruses. Multiplex PCR is less expensive than the singleplex option for simultaneous detection of multiple pathogens from the same clinical sample. However, multiplex PCR often appears to be less sensitive compared to singleplex PCR because it is necessary to optimise more than one pair of primers for PCR amplification. A pre-enrichment of the template cDNA will aid in the detection of pathogens by subsequent PCR amplification.

This study was conducted to determine the performance of RT-Bst for the amplification of virus templates and to improve the sensitivity of PCR-based detection of respiratory viruses from nasopharyngeal samples.

## Materials and methods

### *Nasopharyngeal samples*

After obtaining ethical approval from the National Research Ethics Service (NRES, reference 10/H0808/50), 25 naso-

pharyngeal samples were collected by clinicians during routine examination of hospital patients being investigated for suspected respiratory tract infection. Only excess residual samples were provided after being anonymised. Samples were collected between September 2010 and March 2011 and underwent immediate routine virology tests in the hospital diagnostic laboratory. All samples were tested in the hospital by immunofluorescence (IF) for a panel of viral pathogens (influenza A and B, parainfluenza, respiratory syncytial virus [RSV], human metapneumovirus [MPV] and adenovirus). Ten of these samples were also tested in the hospital by RT-PCR for RSV, influenza A and B, parainfluenza 1–4, MPV, adenovirus and rhinovirus. The samples were later also analysed in this study using RT-PCR and RT-Bst between March and September 2012.

*Extraction of RNA*

Viral RNA was extracted using the QIAamp Viral RNA Mini Kit (Qiagen, Crawley, UK) following the manufacturer's instructions. Carrier RNA was added to the buffer in order to protect RNA from degradation and improve the binding and recovery of small amount of RNA from the column. RNA was eluted with 60 μL AVE buffer. Extracted nucleic acids were used either for one-step RT-PCR and RT-Bst amplification.

*OneStep RT-PCR*

The OneStep RT-PCR kit (Qiagen, Crawley, UK) was used for the detection of 12 common pathogenic viruses of the respiratory tract using three separate multiplex reactions as described elsewhere (Table 1).[18] Multiplex 1 contained RSV, influenza A, influenza B and MPV primers; multiplex 2 contained parainfluenza 1–4 primers; multiplex 3 contained rhinovirus (RV), coronavirus (CoV) 229E, CoV OC43 and influenza C primers, respectively. Equimolar (0.5 μmol/L) concentrations of four sets of primers were added for detection of four viruses in multiplex 1, 2 and 3. A singleplex PCR was set up separately along with all three multiplex

**Table 1.** Primers used for multiplex 1, 2 and 3 for RT-Bst PCR and RT-PCR.

| Virus | Primer name | Sequence (5'→3') | Gene | Amplicon size (bp) | Reference |
|---|---|---|---|---|---|
| ***Multiplex 1*** | | | | | |
| RSV | vrs P1 | 5'-GGAACAAGTTGTTGAGGTT TATGAATATGC-3' | Nucleocapsid | 279 | 18 |
| | vrs P2 | 5'-TTCTGCTGTCAAGTCTAGT ACACTGTAGT-3' | | | |
| Influenza A | mia 1 | 5'-CAGAGACTTGAAGATGTCT TTGCTGG-3' | Matrix protein | 212 | 18 |
| | mia 2 | 5'-GCTCTGTCCATGTTATTTG-3' | | | |
| Influenza B | Mib 1 | 5'-AAAATTACATGTTGGTTCG GTG-3' | Matrix protein | 362 | 18 |
| | Mib 2 | 5'-AGCGTTCCTAGTTTTACT TG-3' | | | |
| MPV | hmpv1 | 5'-CCCTTTGTTTCAGGCCAA-3' | Matrix protein | 416 | 18 |
| | hmpv2 | 5'-GCAGCTTCAACAGTAGCTG-3' | | | |
| ***Multiplex 2*** | | | | | |
| Parainfluenza 1 | PIS1+ | 5'-CCGGTAATTTCTCATACCT ATG-3' | Haemagglutinin-neuraminidase | 317 | 18 |
| | PIS1– | 5'-CCTTGGAGCGGAGTTGTT AAG-3' | | | |
| Parainfluenza 2 | PIP2+ | 5'-AACAATCTGCTGCAGCAT TT-3' | Haemagglutinin-neuraminidase | 507 | 18 |
| | PIP2– | 5'-ATGTCAGACAATGGGCAA AT-3' | | | |
| Parainfluenza 3 | Para 3.1 | 5'-CTCGAGGTTGTCAGGATA TAG-3' | Haemagglutinin-neuraminidase | 189 | 18 |
| | Para 3.2 | 5'-CTTTGGGAGTTGAACACAG TT-3' | | | |
| Parainfluenza 4 | PIP4+ | 5'-CTGAACGGTTGCATTCAG GT-3' | Phosphoprotein | 451 | 18 |
| | PIP4– | 5'-TTGCATCAAGAATGAGTC CT-3' | | | |
| ***Multiplex 3*** | | | | | |
| RV | SRHI1 | 5'-GCATCIGGYARYTTCCACC ACCANCC-3' | VP4/VP2/5'NC | 549 | 18 |
| | SRHI2 | 5'-GGGACCAACTACTTTGGG TGTCCGTGT-3' | | | |
| CoV 229E | MD1 | 5'-TGGCCCCATTAAAAATGT GT-3' | Gene M | 573 | 18 |
| | MD3 | 5'-CCTGAACACCTGAAGCCA AT-3' | | | |
| CoV OC43 | MF1 | 5'-GGCTTATGTGGCCCCTTA CT-3' | Gene M | 335 | 18 |
| | MF3 | 5'-GGCAAATCTGCCCAAGAA TA-3' | | | |
| Influenza C | CHAA | 5'-CHAAACACTTCCAACCCAA TTTGG-3' | Haemagglutinin-esterase | 485 | 18 |
| | CHAD | 5'-CCTGACAGCAACTCCCTC AT-3' | | | |
| ***Internal controls*** | | | | | |
| MS2 | MS2-F | 5'-CTGGGCAATAGTCAAA-3' | MS2 genome | 314 | 20 |
| | MS2-R | 5'-CGTGGATCTGACATAC-3' | | | |

PCR to determine the amplification of a spiked internal control (MS2 RNA).[26] The reaction mix was set up in a volume of 15 μL as described elsewhere.[18]

Briefly, 3.0 μL 5X RT buffer, 0.6 μL dNTP (10 mmol each), 0.75 μL (10 mmol/L) primers (four pairs) for each multiplex reaction (0.75 × 8 = 6.0 μL), 1.8 μL of Q solution and 0.5 μL nuclease-free water, 0.6 μL one-step RT enzyme mix were added to 2.5 μL template RNA for preparation of PCR reaction for detection of viruses of multiplex 1 and 3. The reaction mix for multiplex 2 was prepared in a similar way to multiplex 1 and 3 except that the Q solution was omitted and the total volume made up by adding nuclease-free water.

PCR was initiated by heat activation of HotStarTaq polymerase at 94°C for 15 min. The thermal cycler was programmed for 40 cycles, 94°C for 30 sec, 55°C for 30 sec or 58°C for 30 sec and 72°C for 1 min. A final extension was performed at 72°C for 10 min. The annealing temperature for multiplex 1, 2 and internal control was 55°C whereas the annealing temperature for multiplex 3 was 58°C.[18]

**Table 2.** Detection of 12 respiratory tract viruses in nasopharyngeal samples(1–25) using multiplex one-step RT-PCR and RT-Bst PCR in this study and immunofluorescence (IF) and RT-PCR by the hospital laboratory.

| Sample No. | Hospital results | | Current study | |
|---|---|---|---|---|
| | IF | RT-PCR | One step RT-PCR | RT-Bst PCR |
| 1 | IA | IA (H1N1) | IA (H1N1) | IA (H1N1) |
| 2 | – | – | RSV | RSV |
| 3 | – | n/a† | – | – |
| 4 | –* | n/a | – | RSV |
| 5 | – | – | – | – |
| 6 | – | n/a | – | – |
| 7 | – | n/a | – | – |
| 8 | –* | n/a | – | RSV |
| 9 | – | n/a | RSV | RSV |
| 10 | – | n/a | – | – |
| 11 | – | n/a | – | – |
| 12 | – | – | – | – |
| 13 | – | n/a | – | – |
| 14 | – | – | – | – |
| 15 | – | RSV | RV | RV |
| 16 | – | – | – | – |
| 17 | – | – | – | – |
| 18 | – | – | – | – |
| 19 | – | n/a | – | – |
| 20 | – | n/a | – | – |
| 21 | – | – | – | – |
| 22 | – | n/a | – | – |
| 23 | RSV | n/a | SWA | SWA, RSV |
| 24 | – | n/a | – | – |
| 25 | – | – | – | – |

*Transplant patient; †Data not available; –: negative;
IA: influenza A; RSV: respiratory syncytial virus;
RV: human rhinovirus; SWA: swine influenza virus

*RT-Bst amplification and PCR*

RT-Bst amplification was carried out in a volume of 10 μL as described previously.[22] Nuclease-free water (3.85 μL) was added to 1.0 μL 10X RT buffer, 1.0 μL of 10 μmol/L pentadecamer random primer, 0.5 μL of 10 mmol/L dNTP, 0.25 μL RNase inhibitor (40.0 units/μL, Fermentas, York, UK), 0.5 μL Premium reverse transcriptase (200 units/μL, Fermentas,York, UK), 0.4 μL *Bst* DNA polymerase (8.0 units/μL, Cambridge Biosciences, Cambridge, UK) and 2.5 μL template RNA. The RT-Bst reaction was incubated at 25°C for 10 min (to allow hybridisation of random primers), 50°C for 30 min (for reverse transcription) and 60°C for 1 h for *Bst* DNA polymerase amplification. After incubation the RT-Bst reaction was heat-inactivated at 85°C for 5 min and 1–2 μL cDNA was added to 20 μL of HotStarTaq PCR reaction for detection of 12 common pathogenic viruses of the respiratory tract, as shown in Table 1.

A HotStarTaq PCR reaction was used for the multiplex detection of 12 respiratory tract viruses from RT-Bst-amplified products in three multiplex reactions. For multiplex 1 and 3 reaction mixes, 2.0 μL RT-Bst-amplified products were added to 3.9 μL nuclease-free water, 2.0 μL 10X PCR buffer, 1.2 μL $MgCl_2$ (15 mmol/L), 0.4 μL dNTP (10 mmol/L), 1.0 μL (10 μmol/L) each forward and reverse primer (1.0 × 8 = 8.0 μL), 0.1 μL HotStarTaq DNA polymerase (5.0 units/μL) and 2.4 μL Q solution to make a 20 μL reaction mix. The reaction mix for multiplex 2 was set up in a similar way but water was added instead of the Q solution. The PCR cycling conditions for multiplex 1, 2 and 3 were identical to those of the OneStep RT-PCR reactions shown above. These results were compared directly with those of multiplex one-step RT-PCR which was performed at the same time.

An additional reaction was set up for each one-step RT-PCR and RT-Bst PCR for detection of a spiked internal control, MS2 RNA.[26] MS2 RNA was used to determine the efficiency of RNA extraction, reverse transcription and PCR detection. Additional primer sequences, as recommended by the World Health Organization for the detection of influenza A type H1N1 and swine influenza A, were used to screen all samples using either a singleplex one-step RT-PCR or a singleplex HotStarTaq PCR after RT-Bst amplification for detection of recent influenza strains.

*Sequence analysis*

PCR amplification bands of expected product size were purified from agarose gels using QIA-quick PCR purification kit (Qiagen, Crawley, UK) and the purified DNA was sequenced either using forward or reverse primers previously used for the PCR amplification. In the case where PCR was positive for both one-step RT-PCR and RT-Bst amplification, either one of the PCR products was purified and sequenced. All multiplex PCR-positive reactions were confirmed using a singleplex PCR reaction using corresponding virus-specific primers followed by sequencing the PCR product using the Sanger sequencing method (GATC Biotech, London, UK). All sequences were matched with homologous sequences in the BLASTn database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

*Data analysis*

The significance of agreement between the combined IF/RT-PCR results performed by the hospital laboratory and RT-PCR and RT-Bst performed in this study was compared by

**Table 3.** Primer positions in the virus genomes used for multiplex 1, 2 and 3.

| Virus | Genome (RNA) type | Genome length (bp) | Primer binding site |
|---|---|---|---|
| ***Multiplex 1*** | | | |
| RSV | ss, linear | 15107 | 1953–2229 |
| Influenza A | ss, linear (9 segments) | 996 | 86–279 |
| Influenza B | ss, linear (8 segments) | 1150 | 78–418 |
| MPV | ss, linear | 13335 | 2292–2718 |
| ***Multiplex 2*** | | | |
| Parainfluenza 1 | ss, linear | 15383 | 7530–7846 |
| Parainfluenza 2 | ss, linear | 15646 | 7433–7940 |
| Parainfluenza 3 | ss, linear | 15462 | 7605–7457 |
| Parainfluenza 4 | ss, linear | 17052 | 1969–2410 |
| ***Multiplex 3*** | | | |
| RV | ss, linear | 7152 | 532–1079 |
| CoV 229E | ss, linear | 27208 | 24875–25448 |
| CoV OC43 | ss, linear | 30744 | 28555–28869 |
| Influenza C | ss linear (7 segments) | 1807 | 595–1079 |
| ***Internal control*** | | | |
| MS2 | ss, linear | 3569 | 2717–3031 |

Cohen's kappa test using SPSS. Kappa values of >0.6 and >0.8 were considered indicators of good and almost perfect agreement, respectively.

## Results and discussion

Determining the aetiology of acute viral respiratory tract infections is complex and involves a large number of possible pathogens. In most cases, the causative organism remains unknown, in part due to the lack of a universal and affordable detection process. RT-Bst is an integrated approach for the amplification of single-stranded RNA and DNA templates to improve multiplex detection of pathogens.[22]

In this study, RT-Bst PCR results were compared alongside those of the OneStep RT-PCR kit (Qiagen, Crawley, UK). The results of both were also compared with routine IF and RT-PCR techniques used by the hospital that provided the samples. It is noteworthy here that the RT-Bst PCR and RT-PCR tests were performed several months after the tests were done in the hospital and significant degradation of clinical material may have occurred during this period.

Using routine diagnostic algorithms consisting of IF and RT-PCR, three out of 25 samples (12%) were positive for a viral pathogen according to the hospital laboratory (Table 2). In comparison, one-step RT-PCR detected a pathogen in five out of 25 samples (20%) and RT-Bst PCR detected a pathogen in seven out of 25 samples (28%) including one case of mixed infection (Table 2). The MS2 internal RNA control was amplified in all reactions performed. Sequence analysis of amplified products returned a similarity match of >98% against the expected virus genome by BLASTn in all cases. Reproducibility of these results was confirmed by performing all tests in triplicate on separate days, and identical results were observed each time.

The RT-Bst method was evaluated initially using known virus sequences at fixed and known concentrations.[22] In this clinical evaluation, patient samples were used and consequently there was no gold standard against which the test results could be compared. The Cohen's kappa score was calculated to be 0.66, indicating good agreement between tests, but, in the absence of a standard, these results should be interpreted with caution. While the true burden of viral pathogens in the samples provided was unknown, RT-Bst revealed that greater than two-fold more samples were positive for a viral pathogen compared to the combined IF/RT-PCR test results generated by the hospital laboratory.

RSV sequence was identified in 20% of samples using RT-Bst PCR but only 8% when using one-step RT-PCR. Interestingly, sample 4 was found to be positive for RSV sequence by RT-Bst PCR but not by one-step RT-PCR or by the hospital laboratory. However, RSV was detected by an IF test in the hospital laboratory in a second sample collected five days later. For samples 4, 8 and 23, the presence of RSV was not detected by one-step RT-PCR but was positive by RT-Bst PCR. The failure to detect RSV RNA using one-step RT-PCR was possibly due to the lower abundance of viral RNA in these samples which were enriched by the RT-Bst method. When sample 15 was processed by the hospital laboratory it was shown to be positive for RSV by PCR. RT-PCR and RT-Bst PCR tests performed some months later were negative for RSV but positive for RV sequence. The reasons for this discrepancy are not known.

Viral genomes can be detected even when they are sheared and degraded provided the target sequence remains intact and enough copies are present for amplification. RNA is a labile molecule and degrades easily even during nucleic acid extraction, therefore the RNA extracted from the sample was used immediately (within 30

minutes) in order to obtain relatively longer and intact RNA templates for both RT-Bst amplification and one-step RT-PCR. The RT-Bst amplification method used in this study was capable of amplifying viral RNA of different sizes, including the influenza A genome (996 bp) and RSV genome (15 kb). However, RT-Bst PCR was more effective for the detection of RSV sequences, which is possibly due to the large size of the genome although it cannot be ruled out that the position of primers for PCR detection of RSV was more suitable compared to the other virus sequences.

It has also been shown that the position of the target primer sequence on the genome and the amplicon size are also crucial factors for consideration in the design of RT-PCR assays.[25,27] PCR primers used in this study were selected from published research and designed to amplify the conserved sequence of a particular type of virus; for example, the nucleocapsid gene for RSV and the capsid gene for influenza virus. The information on genome size and location of primer binding sites used in this study is shown in Table 3. These primers were not designed to amplify the central region of the virus sequence and hence they may not be optimal for RT-Bst PCR amplification. During *Bst* DNA polymerase amplification, the pentadecamer primers used to generate cDNA bind randomly throughout the genome and synthesises the complementary strand in a 5′-3′ direction. Different lengths of complementary strands will be synthesised depending on the position of the random primers bound to the template. Primers which bind to the 3′-end of the template are more likely to produce longer templates compared to those which bind further downstream. Due to the repeated amplification of cDNA using random primers and forming a hyperbranched structure of the amplified template, it is possible that the central portion of the template DNA will be amplified more than the terminal portions. This suggests that the selection of a shorter sequence (<250 bp) from the central section of viral genomes of size greater than 2 kb may enhance virus detection following RT-Bst amplification, and that RT-Bst will be useful particularly for the detection of viruses with relatively large genomes, such as RSV.

Based on this study, RT-Bst PCR has the potential to be more sensitive than one-step RT-PCR, as demonstrated by the improved detection of RSV, provided the primers are designed to span the central portion of the genome and extracted RNA is reverse transcribed within 30 minutes. The sensitivity of these results may have suffered due to the delay before they were used in this study, but, despite this, the RT-Bst assay performed as well as, if not better than, RT-PCR and the methods employed currently in routine diagnostic testing. It seems likely that the performance of RT-Bst would be enhanced further when working on freshly collected samples. A comparison of the detection of viruses by the hospital laboratory and this study is shown in Table 2. We also evaluated the costs of consumables and RT-Bst PCR was estimated to be approximately 40% less expensive than RT-PCR, and both methods require only a thermal cycler.

In summary, RT-Bst was found to be potentially a more sensitive and cost-effective method for detection of RNA viruses of the respiratory tract, making this an attractive method for use in diagnostic laboratories, particularly those in developing countries and those with limited access to costly molecular diagnostic instrumentation. However, a further study including a larger number of patient samples is required for further validation of this approach. The RT-Bst protocol needs to be applied to different types of viral genome; for example, single-stranded and double-stranded both linear and circular forms to determine its validity for sequence-independent amplification of different structural conformations of nucleic acids. RT-Bst-amplified products may also be used for subsequent technologies including multiplex PCR-based detection, microarray analysis, hybridisation-based detection and characterisation, cloning and library preparation, and next-generation sequencing for virus discovery. □

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