RT-Bst: an integrated approach for reverse transcription and enrichment of cDNA from viral RNA

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ABSTRACT

The synthesis of cDNA from RNA is challenging due to the inefficiency of reverse transcription (RT). In order to address this, an RT-Bst method was developed for sequential RT of RNA and *Bst* DNA polymerase amplification for enrichment of cDNA in a single-tube reaction. Using genomic RNA from bacteriophage MS2, the yield of cDNA produced by RT alone and RT-Bst were compared by analysis of polymerase chain reaction (PCR)-amplified products. A superior performance was observed when amplifying MS2 cDNA with random primers following RT-Bst compared to RT alone, indicating greater quantities of cDNA were present after RT-Bst. RT-Bst was also compared with RT alone for their relative ability to produce sufficient cDNA to amplify eight target regions spanning the respiratory syncytial virus (RSV) genome. Six out of eight targets were amplified consistently by PCR subsequent to RT-Bst amplification, whereas only three out of eight targets could be amplified after RT alone. The RSV sequences were selectively amplified using RSV-specific primers from a mixed template containing an excess of MS2 RNA without amplifying MS2 sequences. This suggests that RT-Bst can be used to amplify RNA sequences non-specifically using random primers and specifically using sequence-specific primers, and enhances the yield of cDNA when compared to RT alone.

KEY WORDS: Reverse transcription.
RT-Bst amplification.
Virus detection.

Introduction

Obtaining high-quality complementary DNA (cDNA) from RNA is an essential starting point for effective reverse transcription (RT). This is problematical because of the inefficiency of this process, as it is estimated that only 40–80% of template RNA is converted to cDNA during RT.[1] This effectively means that reverse transcription polymerase chain reaction (RT-PCR) detection of a target sequence lacks sensitivity when directly compared to PCR due to the decrease in template concentration during the RT step.[2,3] In a diagnostic setting, this will lead to false-negative results which may compromise patient management and lead to onward transmission of a pathogen. Therefore, there is a clear need to develop methods that can alleviate the problem of losses in template during the process of RT.

DNA polymerases which have a high processivity, such as phi29 and the large fragment of *Bst* polymerase, are being used increasingly in situations where ultrasensitive detection of DNA is required. These enzymes amplify DNA by multiple displacement amplification and can use random primers to non-specifically amplify limiting quantities of DNA. There have been several studies where these polymerases have been used for non-specific amplification of DNA, followed by sequence-specific conventional PCR amplification, and this pretreatment enriches the total DNA to levels that can be amplified by PCR.[4–6] However, such methods are only applicable for DNA targets and there is a need for those which can be applied for studies of RNA targets such as ultrasensitive detection of pathogenic RNA viruses. Utilising the strong processivity of phi29 DNA polymerase, other workers have developed a new approach for isothermal amplification of RNA sequence through reverse transcription and ligation of single-stranded cDNA before phi29 amplification.[7] *Bst* DNA polymerase has also been used for amplification and detection of a short sequence (~200 bp) of DNA and RNA using loop-mediated isothermal amplification (LAMP) and reverse transcription loop-mediated isothermal amplification (RT-LAMP), respectively.[8,9] However, these protocols do not alleviate problems associated with the low efficiency of reverse transcription. Nucleic acid sequence-based amplification (NASBA) and signal-mediated amplification of RNA technologies (SMART) have been used in studies for the amplification of RNA templates using multiple primers and enzymes.[10,11] However, additional reverse transcription steps are required for the synthesis of cDNA prior to PCR amplification.

We have developed and evaluated a method termed RT-Bst that combines reverse transcription of RNA and *Bst* DNA polymerase amplification in a single-tube reaction for enrichment of cDNA prior to PCR amplification. RT-Bst amplification is a versatile technique that can be used with either random or sequence-specific primers for amplification of whole or single-type RNA sequence, respectively. This approach has a number of potential applications including the enhanced detection of pathogenic RNA viruses from clinical material.

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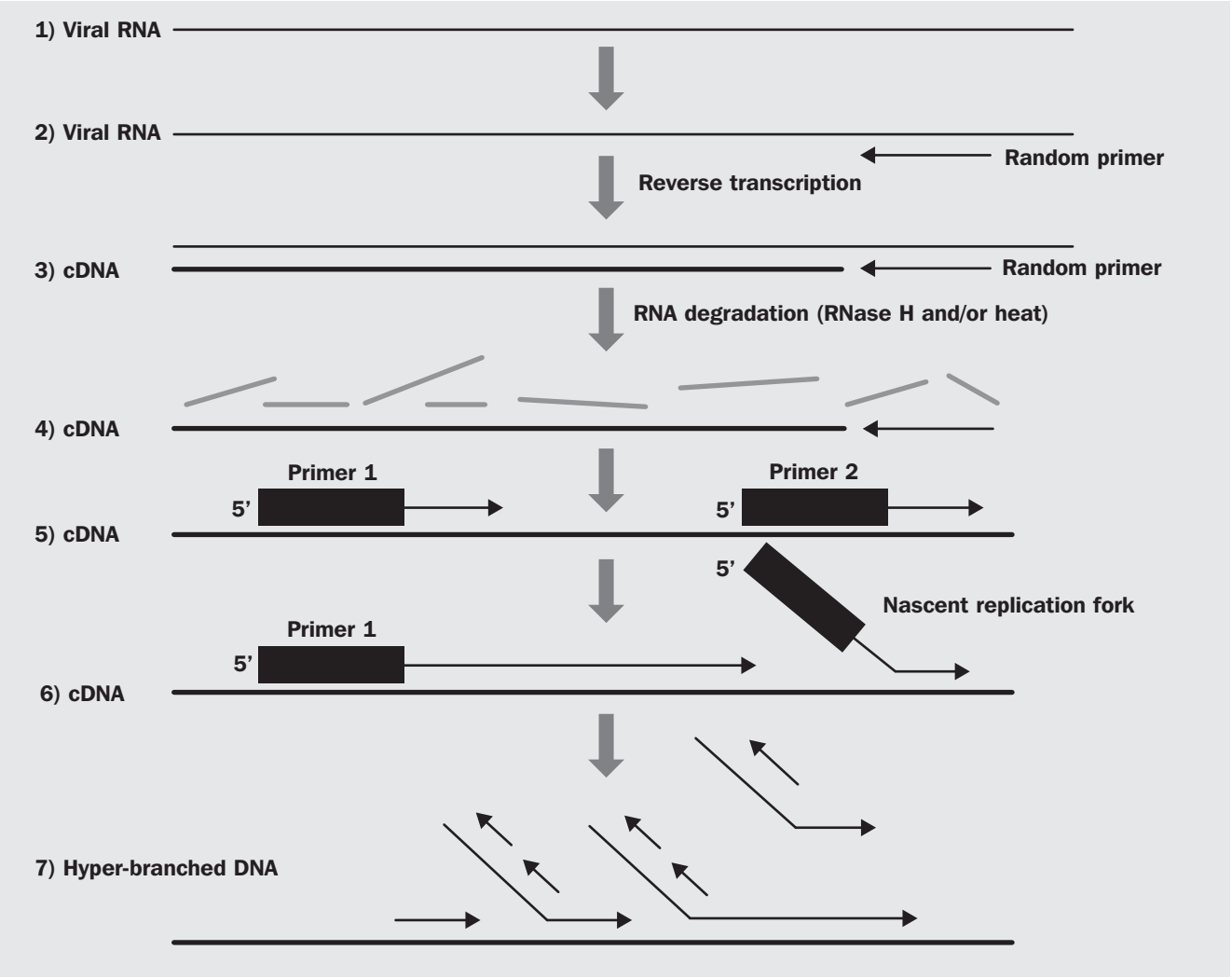

Fig. 1. Model of RT-Bst amplification. **1)** viral RNA; **2)** random primers bound to viral RNA; **3)** synthesis of cDNA by reverse transcription; **4)** RNA degraded by RNase H activity or heat; **5)** random primers bound to single-stranded cDNA; **6)** extension of random primers by *Bst* DNA polymerase; **7)** synthesis of multiple copies of single- and double-stranded cDNA (hyper-branched).

Materials and methods

One-tube RT-Bst method

A method termed RT-Bst was developed by combining reverse transcription and *Bst* DNA polymerase to facilitate the enrichment of cDNA sequences prior to PCR amplification. In this technique, cDNA copies were sequentially generated from RNA templates using reverse transcriptase and then amplified by *Bst* DNA polymerase in a single-tube reaction containing the buffering system supplied for *Bst* DNA polymerase (Fig. 1).

RNA used in this study

Purified bacteriophage MS2 genomic RNA was obtained from DSMZ, Braunschweig, Germany. Genomic RNA of human respiratory syncytial virus (RSV) was extracted from tissue culture fluid positive for RSV using the QIAamp RNA mini kit (Qiagen, Crawley, UK).

PCR amplification assay of MS2 RNA following reverse transcription and RT-Bst amplification

The performance of the QuantiTect Reverse Transcription kit (Qiagen) was compared against RT-Bst for the production of MS2 cDNA using both MMuLV and AMV reverse transcriptase. Two concentrations of MS2 RNA (8 ng/μL and 40 ng/μL) were used for each method. The performance of these two protocols was compared qualitatively from the intensity of PCR bands following agarose gel electrophoresis. Ethidium bromide-stained agarose gels (2% in TAE buffer) were used for separation of amplified PCR products in an electrophoresis tank containing TAE buffer. Amplified products were visualised using a gel documentation system.

The RT-Bst reaction was set up in a 10 μL reaction containing nuclease-free water (4.23 μL), 10x RT buffer (1.0 μL; Fermentas, York, UK), N_{15} random primer (10 μmol/L; 1.0 μL), dNTP (10 mmol/L each; 0.5 μL), RNase inhibitor (40 units/μL; 0.25 μL; Fermentas), MMuLV (200 units/μL) or AMV (10 units/μL) reverse transcriptase (0.12 μL; NEB, Hitchin, UK), Bst DNA polymerase (8.0 units/μL; 0.4 μL (Cambridge Biosciences, Cambridge, UK) and template RNA (2.5 μL).

RT-Bst amplification was performed in a thermal cycler in three steps for reverse transcription of RNA templates and subsequent amplification of cDNA. The RT-Bst reaction was incubated at 25°C for 10 min to allow hybridisation of

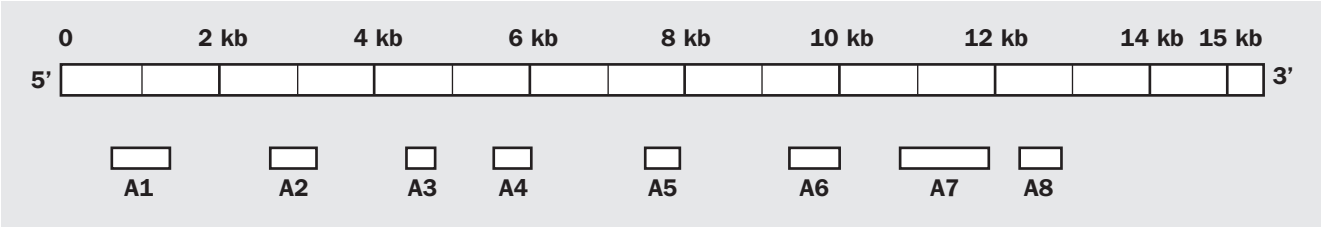

Fig. 2. Schematic diagram of RSV genomic RNA (linear 15,225 bp). A1 to A8 show amplicons corresponding to the eight pairs of primers in Table 1.

random primers, 42°C for 30 min for reverse transcription and 60°C for 1 h for *Bst* DNA polymerase amplification. The reaction was inactivated by heating at 85°C for 5 min. 1–2 μL each RT-Bst amplified and reverse transcribed cDNA was added as template (not more than 10% of reaction volume) in two separate 20 μL HotStarTaq (Qiagen) PCR reactions, following the manufacturer's instructions.

Real-time PCR assay of MS2 RNA following reverse transcription and RT-Bst amplification

Comparisons of reverse transcription of MS2 RNA with RT-Bst were also made using a real-time PCR assay. cDNA was prepared from MS2 RNA using the QuantiTect reverse transcription kit (Qiagen) and by RT-Bst amplification. Both the cDNA products were amplified using the Type-iT HRM kit (Qiagen) containing EvaGreen dye in a Rotor-Gene Q real-time PCR instrument (Qiagen). A three-step PCR cycle was used: 95°C for 5 sec, 55°C for 15 sec and 72°C for 15 sec for 40 cycles. EvaGreen dye fluorescence was recorded in the green channel with excitation at 470 nm and detection at 510 nm.

Primer design

In order to evaluate the ability of RT-Bst to amplify different regions of a genome simultaneously, an assay was performed using RSV genomic RNA (~15 kb), approximately four times larger than MS2 RNA. Eight pairs of primers spanning the entire genome of RSV (GenBank accession number AF013254) were designed using the primer BLAST site from NCBI (www.ncbi. nlm.nih.gov/tools/primer-blast) (Table 1) to be used for RT-Bst amplification. The MS2 RNA amplification primer sequences used were MS2-F (5′-CTGGGCAATAGTCAAA-3′) and MS2-R (5′-CGTGGATCTGACATAC-3′).[12]

Reverse transcription and RT-Bst amplification of the RSV genome using N_{15} random primers

cDNA was prepared from 2.5 μL RSV template RNA and N_{15} random primers using either the Quantiscript reverse transcriptase (Qiagen) or the RT-Bst protocol using MMuLV reverse transcriptase (NEB) to compare their ability to reverse transcribe and amplify each of the eight target sequences. 1 μL cDNA samples following reverse transcription and RT-Bst amplification were used as templates in two sets of 20 μL HotStarTaq (Qiagen) PCR reactions.

RT-Bst amplification of the RSV genome using N_{15} random primers and sequence-specific primers

The ability of RT-Bst to amplify an RNA target from within a mixed RNA template was evaluated using both random and sequence-specific primers. A mixture of two RNA types was used with RSV as target and MS2 as background. This was prepared by mixing target RSV RNA with an excess (10-fold) of MS2 RNA. The RT-Bst amplification assay was performed

Table 1. Primers used for PCR amplification of RSV sequences.

Primer sequences (5'–3')	Primer number and orientation	Primer position	Amplicon size (bp)
5'-ACGGACATGAGACCCCTGTCGA-3'	Primer-1-F	671–692	532
5'-TGCTGGATGACAGCAGCTGATCC-3'	Primer-1-R	1181–1203	
5'-AGGACCCACTTCAGCTCGCGAT-3'	Primer-2-F	2818–2839	363
5'-GTTGGTGGTTCCGCTGACGGA-3'	Primer-2-R	3161–3181	
5'-TGGTGTCGCAAAACCACGCCA-3'	Primer-3-F	4576–4596	149
5'-AGTCCTGGCAGTGCGTTGATTCT-3'	Primer-3-R	4703–4725	
5'-CCACCCCCGAAAACACACCCAA-3'	Primer-4-F	5495–5516	498
5'-CTCTTCTGGCCCGGTTGTTGGC-3'	Primer-4-R	5972–5993	
5'-AGCTGGGGCAAATATGTCGCGA-3'	Primer-5-F	7605–7626	214
5'-TGTCCAGTTCAGCAGCTCCACT-3'	Primer-5-R	7798–7819	
5'-AGCTTAGGGCTGAGATGTGGATTCA-3'	Primer-6-F	9400–9424	565
5'-GCATTCCTTAAAGTGGGCCATCTGT-3'	Primer-6-R	9941–9965	
5'-GGGTGGTATTGAGGGCTGGTGTC-3'	Primer-7-F	10830–10852	920
5'-GACCCTAACGCCTGTGGATCCCT-3'	Primer-7-R	11683–11705	
5'-GCCATGGGTAGGTTCATCTACGCA-3'	Primer-8-F	12288–12311	422
5'-TGTTCCACAACCGACATCAGGCT-3'	Primer-8-R	12688–12710	

F: Forward, R: Reverse

using both random and RSV sequence-specific primers using RevertAid Premium reverse transcriptase (Fermentas). Eight sets of RSV primers were evaluated which amplify different regions of the RSV genome (Table 1, Fig. 2). RT-Bst assays were performed in triplicate using equimolar concentrations (0.5 μmol/L) of sequence-specific or random primers (N_{15}). Following RT-Bst, PCR reactions were set up in fresh tubes using sequence-specific primers (Table 1).

Statistical analysis

Statistical analysis of replicate real-time PCR reactions was performed using SPSS. Ct values of amplified MS2 cDNA prepared by RT and RT-Bst were compared using a two tailed *t*-test. $P<0.01$ was considered statistically significant.

Results

PCR amplification assay of MS2 RNA following reverse transcription and RT-Bst amplification

The RT-Bst protocol for reverse transcription and *Bst* polymerase amplification of bacteriophage MS2 cDNA was shown to enrich the cDNA template concentration reproducibly, compared to that of reverse transcription alone (Fig. 3). Based on the intensity of PCR bands, it was shown that the relative concentration of cDNA was higher when the RT-Bst reaction was performed with MMuLV reverse transcriptase, compared to AMV reverse transcriptase or Quantiscript reverse transcriptase (Fig. 3).

Real-time PCR assay of MS2 RNA following reverse transcription and RT-Bst amplification

Real-time PCR was performed using a Rotor-Gene Q to determine the differences between the cDNA concentrations synthesised by RT-Bst or RT alone based on their normalised fluorescence emission (Fig. 4). The normalisation process is performed by the Rotor-Gene Q software and compensates for differences in background fluorescence between tubes due to slight variations in reaction volume and temperature. Analysis of three replicate real-time PCR runs showed that the RT-Bst amplification method produced a higher concentration of cDNA (average Ct value: 29.4) than did reverse transcription alone (average Ct value: 35.9), and this difference was shown to be statistically significant ($P<0.01$). Interestingly, PCR amplification of a 1 in 10 dilution of RT-Bst-amplified cDNA in nuclease-free water showed a relatively higher average fluorescence (average Ct: 27.0, $P<0.01$) than did the undiluted cDNA.

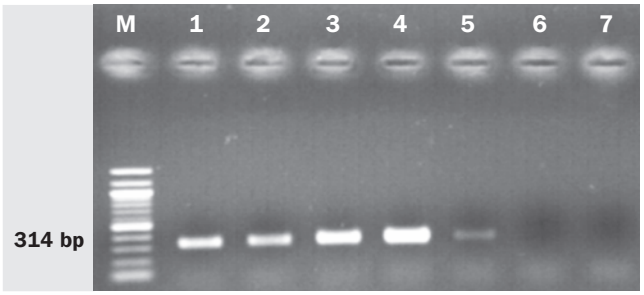

Fig. 3. Single-target PCR amplification assay following RT-Bst amplification of MS2 RNA using different types of reverse transcriptase. **M)** 100-bp DNA ladder; **1** and **2)** RT-Bst amplification using Quantiscript reverse transcriptase; **3** and **4)** RT-Bst amplification using MMuLV reverse transcriptase; **5** and **6)** RT-Bst using AMV reverse transcriptase; **7)** negative control. **1**, **3** and **5)** MS2 RNA (8 ng/μL); **2**, **4** and **6)** MS2 RNA (40 ng/μL); MS2 amplicon size 314 bp.

Reverse transcription and RT-Bst amplification of the RSV genome using N_{15} random primers

The performance of RT-Bst and RT for synthesis of cDNA from the RSV genome using N_{15} random primers was assessed by PCR amplification assay using eight primer sets. Agarose gel electrophoresis of the PCR-amplified products showed that six out of eight sets of primers of RSV were amplified to give a visible product when the RT-Bst protocol

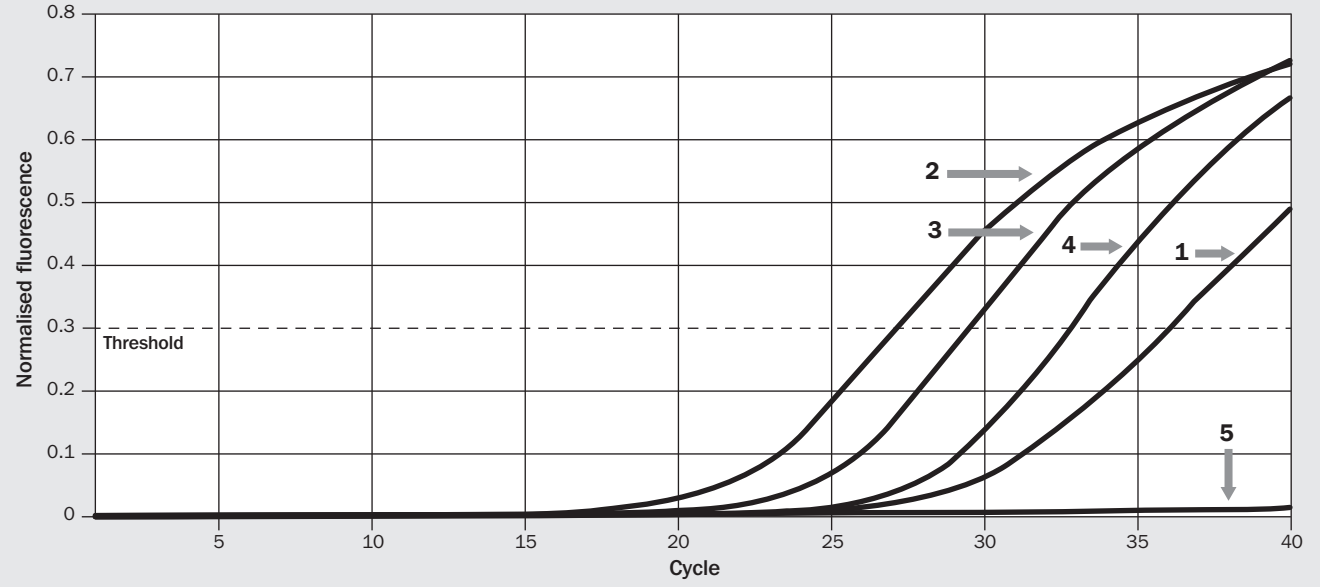

Fig. 4. Real-time PCR amplification of MS2 RNA following reverse transcription and RT-Bst amplification. **1)** reverse transcribed cDNA using the QuantiTect reverse transcription kit (Ct: 35.93); **2)** RT-Bst-amplified MS2 cDNA after 1:10 dilution (Ct: 27.10); **3)** RT-Bst-amplified MS2 cDNA not diluted (Ct: 29.48); **4)** RT-Bst-amplified MS2 cDNA after 1:100 dilution (Ct: 32.73); **5)** No template control (NTC).

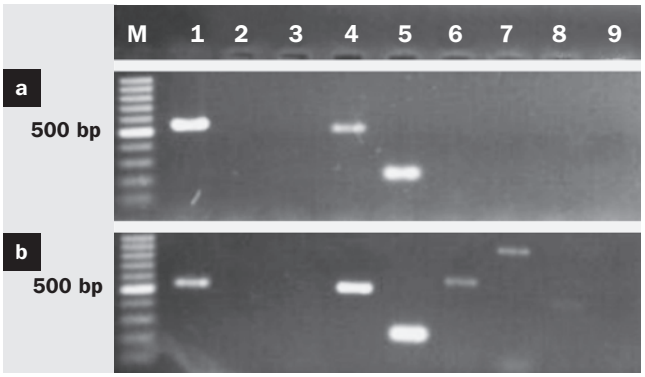

Fig. 5. PCR amplification assay following reverse transcriptase and RT-Bst amplification of RSV genomic RNA. **a)** Reverse transcription using Quantiscript reverse transcriptase; **b)** RT-Bst amplification using MMuLV reverse transcriptase; **M)** 100-bp DNA ladder; **1–8)** PCR detection of eight different regions of the RSV genome (Table 1, Fig. 2); **9)** negative control.

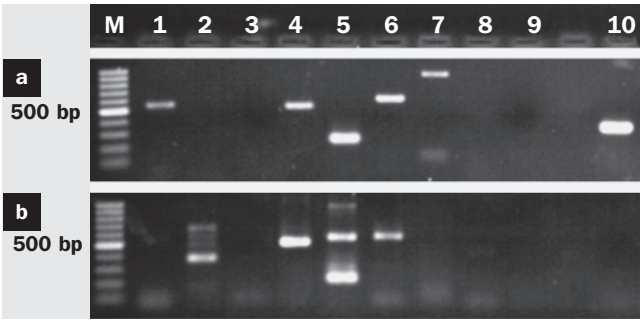

Fig. 6. Eight target PCR amplification assay following RT-Bst amplification of the RSV genome from a mixed template using random and sequence-specific primers with RevertAid Premium reverse transcriptase. **a)** RT-Bst amplification using N_{15} random primers; **b)** RT-Bst amplification using eight pairs of RSV genome-specific primers; **M)** 100-bp DNA ladder; **1–8)** PCR detection for eight different fragments of RSV using eight pairs of primers (Table 1); **9)** negative control; **10)** MS2 cDNA.

was used for cDNA preparation (Fig. 5B). In contrast, only three out of eight pairs of primers of RSV gave visible products when cDNA was synthesised using the Quantiscript reverse transcriptase (Fig. 5A). This assay was conducted in triplicate and produced identical results each time.

RT-Bst amplification of the RSV genome using N_{15} random primers and sequence-specific primers

The ability of RT-Bst to generate and amplify RSV cDNA in the presence of a 10-fold excess of MS2 RNA was determined using random and sequence-specific primers. It was shown that five out of eight pairs of primers produced amplicons in both the RT-Bst assays when cDNA was prepared using sequence-specific (Fig. 6B) and N_{15} random primers (Fig.6A). The same results were obtained in three consecutive experiments. It was also found that MS2 sequences were only amplified when random primers were used (Fig. 6A, Lane 10).

Discussion

An assay which can compensate for the inefficiency of reverse transcription would go some way to improve the ability to detect mRNA and pathogenic RNA viruses. The development of an isothermal one-tube RT-Bst method allows reverse transcription and amplification of cDNA from RNA using a simple instrument such as a heating block or water bath. Alternative isothermal methods can be used to amplify viral RNA genomes, such as RT-LAMP,[9] NASBA[13,14] and SMART.[15] However, some features of the RT-Bst method are novel and clearly distinguish it from other isothermal methods. NASBA and SMART directly amplify RNA while RT-Bst synthesises cDNA from RNA and amplifies cDNA in a single reaction tube, eliminating the need for an additional RT step. RT-LAMP amplifies a short fragment (~200 bp) of cDNA, whereas RT-Bst can amplify the whole genome sequence of single-stranded RNA or DNA. As cDNA is more stable than RNA, this makes it a more convenient template for the detection of viruses and preservation of cDNA templates for downstream analysis.

The single-target PCR amplification assay of MS2 RNA following RT-Bst amplification demonstrates that the MMuLV reverse transcriptase is more efficient for RT-Bst amplification than AMV reverse transcriptase (Fig. 4). However, PCR amplification of MS2 cDNA sequences following RT-Bst amplification and reverse transcription using only one primer pair did not confirm representative amplification of the entire genome. Consequently, an alternative assay was performed to determine representative and genome-wide amplification.

Comparison of the RT and RT-Bst protocols in their relative ability to amplify eight regions spanning the RSV genome confirmed that the performance of the RT-Bst protocol is more efficient than the Quantiscript reverse transcriptase kit (Fig. 5). This indicates that RT-Bst could be used as an alternative protocol to reverse transcription alone for genome-wide amplification of RSV and other RNA genomes. While the results indicate that RT-Bst is superior to reverse transcription alone, the reasons for some primer sets failing to yield a product in the RT-Bst assay (Fig.5, Lanes 2 and 3) could not be determined. However, this could be due to secondary structures within the RSV RNA genome. Web-based software was used for the determination of secondary structures within the RSV RNA genome (http://rna.urmc.rochester.edu/RNAstructureWeb/Servers/Predict1/Predict1.html). This showed that greater degrees of secondary structures were present in the region amplified by regions A2 and A3 (Fig. 2, Fig. 5, lanes 2 and 3) which failed to yield a product by RT-Bst (data not shown) and therefore this needs to be considered during primer design.

RT-Bst amplification of the RSV genome from a mixed RNA template using N_{15} random primers and RSV sequence-specific primers suggests that these approaches can be used for random and selective amplification of pathogenic RNA virus sequences, respectively. Therefore, MS2 sequences could be co-amplified with RSV sequences in an RT-Bst reaction only when N_{15} random primers are used on mixed RNA templates. Sequence-specific and random primer-mediated RT-Bst amplification of RSV sequences showed the amplification of products of different sizes (214–920 bp) in the subsequent PCR amplification assay (Fig. 6). Multiple bands were most likely generated due to the carry-over of

excess primers from the RT-Bst amplification step, which were then added to the PCR reaction mix and amplified.

Following evaluations, RevertAid Premium reverse transcriptase (Fermentas) was used for the RT-Bst assay as it is the thermostable form of MMuLV reverse transcriptase which lacks RNase H activity due to incorporation of a point mutation in the RNase H domain of the MMuLV reverse transcriptase. This point mutation enables this enzyme to produce full-length cDNA of up to 20 kb. The RT-Bst protocol begins with a 50°C step for reverse transcription, followed by a 60°C step for *Bst* DNA polymerase amplification, so any double-stranded DNA present in the reaction will not be denatured at these temperatures and hence will not be amplified. In clinical samples, any single-stranded DNA and RNA from the host could be co-amplified in the RT-Bst reaction when N_{15} random primers are used. The use of sequence-specific primers in the RT-Bst reaction can overcome this problem by selectively amplifying the viral sequence. This finding suggests that both the selective and non-selective RT-Bst approaches for amplification of genomic sequences could be useful for downstream applications such as genotyping viruses from mixed and single-type populations of RNA, respectively.

No inhibition of PCR amplification was observed when RT-Bst-amplified MS2 cDNA was used directly as template up to 10% of the total volume in a block PCR reaction. This result suggests that the quality of cDNA prepared by the RT-Bst reaction is comparable to that of the Quantiscript reverse transcriptase. RT-Bst-amplified cDNA was also used as a template in real-time PCR reactions containing up to 10% of the reaction volume, but this showed inhibition of real-time PCR amplification. However, a 1 in 10 dilution of the RT-Bst-amplified cDNA template in nuclease-free water improved real-time PCR amplification curves, possibly by dilution of random oligonucleotides and buffer carried over from the RT-Bst step, which may interfere with the real-time PCR reaction.

The NASBA and SMART techniques work optimally at temperatures of 41°C and 42°C, respectively. The RT-Bst amplification method developed in this study can be performed at 42°C using MMuLV or at 50°C using RevertAid Premium reverse transcriptase. The higher optimum temperature of RT-Bst amplification could be more effective at eliminating secondary structures during reverse transcription. Based on these findings, the RT-Bst method may be a more appropriate approach than commercial reverse transcription kits for the preparation of cDNA.

Although this study was limited to a pilot scale, RT-Bst appeared to demonstrate improved performance when compared to reverse transcription alone for amplification of single and multiple targets of MS2 and hRSV genome sequences, respectively. Although a larger scale study will be required for appropriate validation, including the quantification of data to determine the relative sensitivity of the method, the RT-Bst protocol has potential for commercialisation and use in diagnostic settings. □

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