Comparison of Three RNA Amplification Methods as Sources of DNA for Sequencing

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ABSTRACT

DNA products generated from a region of the measles virus genome by three RNA reverse transcription and amplification methods were cloned and sequenced, and the results were compared in order to evaluate the methods' relative fidelities. The methods were: (i) *reverse transcription followed by a nested polymerase chain reaction (RT-nPCR),* (ii) *a combined RT-PCR using r*Tth *polymerase and* (iii) *nucleic acid sequence-based amplification (NASBA). NASBA was followed by RT-PCR with r*Tth *polymerase or RT using AMV reverse transcriptase to generate DNA products for cloning. Products from all three sets of reactions were cloned into a vector, pT7Blue, and 790 bp of cloned DNA were sequenced and analyzed for base changes to determine the error rates for each amplification method. Sequence analysis of cloned RT-nPCR products showed no errors, whereas cloned r*Tth *mediated RT-PCR products possessed an error rate of 0.38% and cloned NASBA products 0.38%. Products generated by NASBA followed by RT-PCR with r*Tth *polymerase possessed an error rate of 1.9%. The results indicated that cloned DNA products generated by RT-nPCRs possessed least errors and that for NASBA, RT of reaction products before cloning and sequencing was preferable to using RT-PCR.*

INTRODUCTION

To confirm amplification reaction specificity and to characterize nucleic acid products, reaction products are often sequenced. For example, sequencing individual amplification products generated from RNA or DNA detection methods enables the sequence variation within a particular RNA or DNA sample to be determined, providing valuable information concerning the quasi-species distribution within a given infected clinical sample (8).

Since incorporation errors are often generated by enzymes involved with nucleic acid amplification (5,9,12), it is also desirable that a number of products are sequenced to obtain a good consensus sequence. To achieve this, it is frequently the case that individual amplification products are cloned into a plasmid vector and stabilized in a host bacteria.

We compared the relative fidelities of three different RNA amplification strategies used for producing DNA for sequencing. The methods were *(i)* reverse transcription (RT) using Moloney murine leukemia virus (M-MLV) reverse transcriptase followed by a nested polymerase chain reaction (nPCR) using *Taq* DNA polymerase (10), *(ii)* a combined RT-PCR mediated by r*Tth* polymerase (7) and *(iii)* nucleic acid sequence-based amplification (NASBA) using avian myoblastosis virus (AMV) reverse transcriptase and T7 RNA polymerase (3,4).

In the case of RT-PCR of RNA, DNA products can be cloned directly, whereas with NASBA single-stranded RNA products must first be converted to double-stranded DNA before cloning. Individual clones of such transformed bacteria can be isolated and their recombinant plasmids purified.

Table 1. Positions and Sequences of PCR Primers, NASBA Primers, Target Sites and Sequence of the Internal Probe AB10.

Primer	Position	Sequence[a]
MV1 (PCR) (Upstream/Outer)	1198–1219	TTAGGGCAAGAGATGGTAAGG
MV2 (PCR) (Downstream/Outer)	1610–1631	GTTCTTCCGAGATTCCTGCCA
MV3 (PCR) (Upstream/Inner)	1248–1269	AGCATCTGAACTCGGTATCAC
MV5 (PCR) (Downstream/Inner)	1348–1368	GTTCGGTTCATAGTAAAGA
MV4 (PCR) (Downstream/Inner)	1480–1501	AGCTCTCGCATCACTTGCTCT
AB20 (NASBA) (Upstream)	1200–1219	AGGGCAAGAGATGGTAAGGA
AB22 (NASBA) (Downstream)	1358–1379	**AATTCTAATACGACTCACTATAGGG** GATCACCGTGTAGAAATGACA

PCR primers MV1 and MV2 give a first-round product of 433 bp. PCR primers MV3 and MV4 give a second-round product of 253 bp. NASBA primers AB20 and AB22 give an RNA product of 179 bases. Bold type shows T7 promoter region of primer (1,2).

[a]Orientations of sequences shown are 5′→3′.

MATERIALS AND METHODS

RT-nPCR

RT was performed using M-MLV Reverse Transcriptase (Life Technologies, Paisley, Scotland, UK). One hundred nanograms of total RNA from measles-infected Vero cells (1) were added to 10 µL of RT reaction buffer containing 50 mM Tris-HC1, pH 8.3, 75 mM KC1, 10 mM dithiothreitol (DTT), 3 mM $MgC1_2$, 0.5 mM each dNTP (Advanced Biotechnologies, Epsom, England, UK), 0.75 µM downstream primer MV2 (Oswell DNA Services, Southampton, England, UK), 2 U RNase Inhibitor (Life Technologies) and 200 U M-MLV Reverse Transcriptase. The reaction mixture was then overlaid with 40 µL of mineral oil and incubated at 37°C for 2.5 h. Table 1 shows primer sequences and annealing

Table 2. Error Rates and Base Changes Found for Each of the Three Amplification Methods

	RT-nPCR *Taq* Polymerase	RT-PCR r*Tth* Polymerase	NASBA	NASBA = RT-PCR r*Tth* Polymerase
Error rate	0%	0.38%	0.38%	1.9%
Base Changes		A → T C → T G → A	A → T G → C G → T	A → G (3) A → T C → G C → T G → C T → A (2) T → C (4) T → G (2)

positions.

Two microliters of the above RT reaction were added to 48 µL of a first-round PCR containing 20 mM Tris-HC1, pH 8.4, 50 mM KC1, 0.5% W-1 detergent, 1.5 mM $MgCl_2$, 0.2 mM each dNTP (Advanced Biotechnologies), 0.25 µM of each outer primer (MV1 and 2 [Oswell DNA Services]) and 1.75 U *Taq* DNA Polymerase (Life Technologies). The reaction mixture was then overlaid with 60 µL of mineral oil and thermally cycled in a Model PHC-3 Thermal Cycler (Techne, Cambridge, England, UK). This involved an initial denaturation step of 94°C for 2 min followed by 35 cycles of 94°C for 30 s (denaturation), 56°C for 1 min (primer annealing) and 72°C for 30 s (extension). This was followed by a final extension at 72°C for 5 min.

One microliter of the first-round reaction mixture was added to 24 µL of second-round PCR buffer made up with the same reagent concentrations but with an inner, nested primer pair MV3 and MV4 in place of MV1 and MV2. The reaction mixture was overlaid with 40 µL of mineral oil and amplified for 25 more cycles using the protocol described previously (1).

r*Tth* Mediated RT-PCR

One hundred nanograms of total RNA from measles infected Vero cells (see previous description) were added to 40 µL of r*Tth* reaction mixture containing 50 mM bicine, pH 8.2, 115 mM potassium acetate, 8% glycerol, 2.5 mM manganese acetate, 3 mM each dNTP (Advanced Biotechnologies), 0.45 µM each primer (MV3 and MV4) and 5 U r*Tth* (PE Applied Biosystems, Warrington, England, UK). The reaction mixture was overlaid with 60 µL of mineral oil and incubated at 60°C for 30 min to allow for RT. This was followed by denaturation at 94°C for 2 min and then 40 cycles of 94°C for 1 min (denaturation), 56°C for 1 min (annealing/extension) and a final extension at 60°C for 7 min (1,2).

NASBA

One hundred nanograms of total RNA from measles-infected Vero cells (see previous) were added to 10 µL of NASBA buffer containing (per 20 µL reaction) 40 mM Tris-HC1, pH 8.5, 12 mM $MgC1_2$, 70 mM KC1, 5 mM DTT, 1 mM each dNTP, 2 mM ATP, 2 mM UTP, 2 mM CTP, 1.5 mM GTP, 0.5 mM ITP and 1 µM of each primer (AB20 and AB22, Table 1). The mixture was incubated at 65°C for 5 min and then 41°C for 5 min. A 5-µL volume containing the following enzyme mixture was subsequently added; 0.08 U RNase H, 32 U T7 RNA Polymerase (both from Amersham Pharmacia Biotech, Milton Keynes, England, UK) and 6.5 U AMV Reverse Transcriptase (Takara Biomedicals, Seigagaku, Japan). The reaction mixture was cen-

trifuged briefly at 10 000× *g* and then incubated at 41°C for 1.5 h (1).

Cloning of PCR Products

Measles PCR products were electrophoresed in a 1% (wt/vol) low-melting-point agarose gel (BioGene Ltd, Cambridge, England, UK). A gel slice containing the PCR product was excised and DNA was extracted using the Wizard® PCR Preps DNA Purification System (Promega, Southampton, England, UK). Gel-purified PCR products were cloned into the *Nsi*I site of plasmid pT7Blue T-Vector (Novagen/AMS Biotechnology, Witney, England, UK) using T4 DNA Ligase (Life Technologies) according to the manufacturer's instructions.

Recombinant plasmids were transformed into *E. coli* NovaBlue Competent Cells (Novagen/AMS Biotechnology) and subsequently cultured according to the manufacturer's instructions.

Ten white clones from each transformation were checked for the presence of insert DNA by digestion of plasmid DNA, recovered using the Wizard Miniprep Kit (Promega) with *Nsi*I (insert DNA contained a unique *Nsi*I site). Five micrograms of recombinant plasmid DNA were precipitated with 0.1 vol of 5M sodium acetate and 2 vol of ice-cold ethanol. The pellet was washed three times with 70% (vol/vol) aqueous ethanol before resuspension in 10 µL of sterile double-distilled water (ddH_2O).

Cloning of NASBA Products

NASBA RNA products were converted to cDNA by two methods before cloning: *(i)* by RT-PCR using *rTth* and *(ii)* by RT (without PCR) using a Copy Kit™ (Invitrogen NV, Leek, The Netherlands). In both cases, the NASBA reaction mixture was treated with phenol/chloroform to remove protein and RNA precipitated by addition of 0.6 vol isopropanol before use in RT or RT-PCR. The RNA was collected by centrifugation at 12 000× *g* for 10 min and the pellet resuspended in sterile ddH_2O.

(i) One nanogram of purified NASBA product was used in a *rTth* mediated RT-PCR as described previously but with primers MV3 and MV5 (Table 1). Product identity and size was evaluated by agarose gel electrophoresis. Subsequently, the cDNA was gel-purified (see previous description) and cloned as described in Cloning of PCR Products.

(ii) Five micrograms of purified NASBA product were used together with the Copy Kit to prepare cDNA according to the manufacturer's instructions. Subsequently, the reaction mixture was extracted with phenol/chloroform, and DNA was precipitated by addition of 0.1 vol of 3 M sodium acetate (pH 5.0) and 2.5 vol of ice-cold ethanol followed by incubation at -20°C for 16 h. After centrifugation at 12 000× *g* for 15 min at 4°C, the supernatant was discarded, the DNA pellet washed 2× in 70% (vol/vol) aqueous ethanol and resuspended in 20 µL of sterile ddH_2O. The 5′ termini of the blunt-ended cDNA were then phosphorylated by incubating 10 pmol of purified cDNA in a 50-µL reaction containing T4 Polynucleotide Kinase reaction buffer, 4 mM ATP and 15 U T4 Polynucleotide Kinase for 5 min at 37°C. Subsequently, reactions were terminated by adding 2 µL of 0.5 M EDTA, pH 8.0. Reaction mixtures were treated with phenol/chloroform, and phosphorylated products were precipitated and cloned as described previously in the Cloning RT-PCR Products section.

Sequencing

Sequencing was performed using a Thermo Sequenase™ Cycle Sequencing Kit (Amersham Pharmacia Biotech, Little Chalfont, Bucks, England, UK) according to the manufacturer's instructions. DNA from 10 clones generated by each method was prepared and the 79-bp segment of each clone that was located between primers MV3 and MV5 was sequenced and analyzed for base changes. A total of 790 bases for each method were analyzed.

RESULTS AND DISCUSSION

In a total of 790 bp of DNA sequenced for each amplification method from cloned reaction products, the RT-PCR method using *Taq* DNA polymerase was observed to have generated no base changes, whereas both RT-PCR using r*Tth* polymerase and NASBA followed by RT, generated 3 base changes (error rate = 0.38% for both). However, a total of 15 base changes (error rate = 1.9%) following NASBA and RT-PCR using r*Tth* polymerase were observed. The difference in error rate observed for NASBA products cloned following RT-PCR and RT alone was found to be statistically significant ($P = 0.009$) using a chi square test. Table 2 summarizes the error rates and indicates specific errors produced by the four methods.

These results show that RT-PCR using M-MLV reverse transcriptase and *Taq* DNA polymerase generates fewer errors than either RT-PCR using r*Tth* polymerase or NASBA. The observed error rate of 0.38% for NASBA followed by RT is significantly higher than that reported by Sooknanan et al. (0.03%) (11). Furthermore, unlike Sooknan's findings, no deletions or insertions were observed in this study.

A possible explanation for the high error rate observed in sequences of DNA produced by NASBA followed by r*Tth*-mediated RT-PCR could be the inclusion of inosine triphosphate (ITP) in the NASBA reaction mixture. ITP is included to improve the efficiency of NASBA reactions and, as a result, NASBA reaction products can possess ITP. Because inosine can base pair with all four bases (6), it is possible that when NASBA products are used as templates for r*Tth*-mediated RT-PCR, the enzyme might incorporate any base when it reaches an inosine position in the template sequence. These 'base changes' are detected following cloning and sequencing.

The large numbers of errors generated during NASBA followed by RT-PCR with *rTth* polymerase indicates that this approach to cloning and sequencing NASBA products should only be used when large numbers of cloned reaction products are available for analysis to generate an accurate consensus sequence. Although recombinant plasmid vectors containing NASBA products may be useful for long-term storage and in vitro transcription and translation studies, this approach is not an ideal method for generating error-free DNA for sequencing. Conversely, reverse transcription of NASBA products to generate double-stranded cDNA for direct cloning without amplification leads to fewer errors reported in their sequences.

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