TECHNICAL NOTE

Effect of inhibitors in clinical specimens on *Taq* and *Tth* DNA polymerase-based PCR amplification of influenza A virus

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Fifteen randomly selected nasopharyngeal (NP) swab specimens (culture-negative for influenza A virus) were spiked with influenza A virus and the nucleic acids were extracted and subjected to PCR amplification with *Thermus aquaticus* (*Taq*) and *T. thermophilus* (*Tth*) DNA polymerases. Products of the expected size, and giving equivalent band intensities, were obtained from four specimens with both polymerases. For six specimens, less products were obtained with *Taq* DNA polymerase than with *Tth* DNA polymerase. Products were detected from five NPs only by PCR with *Tth* DNA polymerase. The transport medium and the calcium alginate swab fibre of the specimens were shown not to be the source of the inhibitors. The incorporation of ³²P-dCTP into cDNA, and the yield of PCR products of cDNA made from control RNA template (purified from H₂O spiked virus suspension) were decreased in the presence of inhibitory extracts, showing that both the reverse transcription (RT) and PCR steps in amplification with *Taq* DNA polymerase were sensitive to the inhibitors. In contrast, *Tth* DNA polymerase was more resistant to the inhibitors and viral nucleic acid from all the specimens examined could be amplified and detected in a single step by RT-PCR with *Tth* DNA polymerase.

Introduction

Influenza A virus, a member of the Orthomyxoviridae family [1], is a major causative agent of a broad-spectrum respiratory illness in man [2]. A diagnosis of the viral infection is essential to provide proper therapeutic steps that can reduce the progression as well as severity of illness and a rapid diagnosis can be crucially important for better management of patients, particularly those at high risk. In addition, a rapid diagnosis can help eliminate the chances of inappropriate therapy and facilitates surveillance and control of transmission of the disease. The standard laboratory method for diagnosis of influenza A infection is based on isolation of the virus in tissue culture [3]. However, the tissue culture method is tedious and very time consuming and it is virtually impossible to make an early therapeutic intervention based on detection of the virus by this method. The enzyme-linked immunosorbent assay (ELISA) and immunofluorescence assay

(IFA) have been described for rapid detection of the infection [4]. The polymerase chain reaction (PCR) provides another approach that allows rapid detection of influenza virus and other viral pathogens [5–7] and has been shown to be more sensitive than the standard virological method [5].

Amplification of a target RNA, such as the genome of influenza A virus, involves reverse transcription of RNA to cDNA by the enzyme reverse transcriptase (RT) and subsequent PCR amplification of cDNA by a thermostable DNA polymerase. In previous PCR studies with influenza A virus, *Taq* DNA polymerase (from *Thermus aquaticus*) has been used for cDNA amplification [5, 6]. However, this *Taq* DNA polymerase-based amplification method has been reported to produce false negative results for some clinical specimens [8, 9] because of the presence of inhibitors.

An alternative to *Taq* DNA polymerase-based PCR could be that based on the *Tth* DNA polymerase, a relatively sturdy enzyme [10] from the thermophilic bacterium *T. thermophilus*. The *Tth* enzyme has both RT and DNA polymerase activity. Therefore, a convenient PCR amplification of RNA is possible in

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a single reaction mixture with *Tth* DNA polymerase. An optimised single tube RT-PCR procedure for amplification and detection of influenza A virus with *Tth* enzyme has been reported [11]. The present study compared *Taq* DNA polymerase-based PCR following reverse transcription, with the *Tth* enzyme-based RT-PCR amplification for influenza A virus detection in nasopharyngeal (NP) specimens.

Materials and methods

Virus strain and media

Influenza virus A (H1N1) was obtained from the Department of Public Health, San Diego, CA, USA. The titre of the concentrated stock was 4×10^4 TCID₅₀/ml. The virus strain was cultured, typed and propagated at the virology laboratory of the Department of Public Health, San Diego. The virus was diluted in three media: (1) diethyl pyrocarbonate (DEPC)-treated distilled H₂O (control medium); (2) veal infusion broth (VIB) (transport medium); and (3) DEPC-treated H₂O incubated overnight with a fresh calcium alginate swab. Ten-fold dilutions to 1 in 10^6 of the influenza A virus (H1N1) stock were prepared in each medium.

Clinical specimens

Nasopharyngeal (NP) swab specimens were obtained from the clinical virology laboratory, University of California at San Diego (UCSD) Medical Center. The NP specimens used in this study were all negative for influenza A virus by culture, and also both *Taq* and *Tth* DNA polymerase-based PCR. The NP specimens, stored at -70°C , were thawed and a dilution series of influenza A virus (H1N1) was prepared as in the control medium, in each of the specimens.

Nucleic acid target preparation

The RNA was isolated from 200 μl of influenza viral suspension in medium or in NP specimens with a QIamp kit (Qiagen Inc, Chatsworth, CA, USA) and following the protocol provided by the manufacturer. The RNA was eluted in 100 μl of 10 mM Tris-HCl (pH 9.0). The extracted RNA solution was stored at -20°C and used for PCR amplification within 1 week.

Primers

The sense primer (5'-CCGAGATCGCAGCAGAGACTTGAAGAT3'), and the antisense primer (5'-GGCAAGTGCACCAGCAGAATAACT3'), derived from the conserved regions in the coding sequence of the matrix protein gene of influenza A virus genome, were identical to those used in a previous study [5], and were purchased from Life Technologies, Gaithersburg, MD, USA.

cDNA synthesis and PCR with Taq polymerase

cDNA synthesis was performed in a reaction mixture containing 10 μl of target RNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.25 mM MgCl₂, 0.05 mM of each dNTP (Promega), 20 U of RNasin (Promega), 100 U of Moloney murine leukaemia virus reverse transcriptase (MMLV-RT) (Promega) and 250 μg of random primers (Promega) in a total volume of 20 μl . The cDNA synthesis conditions consisted of one cycle of 10 min at 25°C , 20 min at 42°C followed by 5 min at 95°C (for inactivating the remnants of reverse transcriptase) in a thermocycler 2400 (Perkin Elmer Cetus, Norwalk, CT, USA). The reaction was then stored at 4°C until used for PCR. The 20- μl cDNA reaction mixtures were then added to 30 μl of PCR reaction mixture such that final concentrations of the reaction components were as follows: $1 \times$ Perkin Elmer PCR buffer II (10 mM Tris-HCl, pH 8.9; 50 mM KCl; gelatin 0.01%), 2.5 mM MgCl₂, 200 μM each of dATP, dCTP, dGTP and dUTP, 0.5 μM each of the sense and antisense primers, 0.5 U of Uracil N-glycosylase (UNG) and 1.25 U of *Taq* DNA polymerase (Perkin Elmer). The amplification reactions were performed in the thermocycler 2400 with a precycle of 25°C for 10 min, for UNG digestion of any carried over DNA contaminants, followed by incubation at 95°C for 10 min to inactivate UNG. Amplification conditions consisted of 40 cycles of 94°C for 10 s, 60°C for 15 s and 72°C for 30 s.

RT-PCR by Tth polymerase

Target RNA (10 μl) was amplified in a 50- μl reaction mixture containing 300 μM of each dNTP, 500 μM dUTP, 0.5 μM each of the sense and antisense primers, 0.5 U of UNG, 2.5 U of *Tth* DNA polymerase (Perkin Elmer) and 3.0 mM Mn(OAc)₂, in $1 \times$ PCR buffer for *Tth* enzyme (Perkin Elmer). The precycle conditions consisted of incubation for 10 min at 25°C for digestion of carried over DNA by UNG, 2 min at 95°C for UNG inactivation, 30 min at 60°C for reverse transcription and 3 min at 94°C for removal of secondary structure of cDNA. The amplification conditions were as described above.

Positive and negative PCR controls

Control RNA (10 μl out of 100 μl) isolated from 200 μl of 40–400 TCID₅₀/ml of influenza A virus suspension in DEPC-treated water was used as the positive control for PCR. DEPC-treated H₂O (10 μl) and a non-specific target RNA (PAW109 RNA, Perkin Elmer Cetus) were used as the negative controls for the PCR.

Electrophoresis in agarose gels

Final reaction products (10 μl) were analysed by electrophoresis in TBE buffer (89 mM Tris-borate, 2 mM EDTA, pH. 8.3) in an agarose (2%) gel containing ethidium bromide 1 $\mu\text{g}/\text{ml}$. After electro-

phoresis the DNA bands were visualised with a UV transilluminator.

Reverse transcription (RT) assay

A mixture (10 μ l) containing 20 mM Tris-HCl (pH 8.3), 100 mM KCl, 2.5 mM MgCl₂, 40 U RNasin (Promega), 10 pmol antisense primer, 5 μ l of control RNA or 5 μ l of control RNA plus 1 μ l of RNA extracted from NP specimen no. 5 spiked with a 1 in 10³ dilution of stock virus and diluted five-fold to 1 in 625, was incubated at 70°C for 10 min. Reactions were initiated by the addition of a 10- μ l mixture containing 100 μ M each of dATP, dGTP, dTTP and α -³²P dCTP (3.0 Ci/mmol) and 100 U of MMLV-RT. The final reaction mixture was incubated at 42°C for 30 min and the reaction was terminated by incubating at 95°C for 10 min. Ten μ l of the reaction mixture were then added to 500 μ l of trichloroacetic acid (TCA) 10% and incubated in ice for 30 min. Unincorporated α -³²P dCTP was removed by passing the mixture through a membrane filter of pore size 0.45 μ m (Millipore) and the incorporation of ³²P-labelled dCTP in the synthesised cDNA was determined from TCA precipitable counts detected by Cerenkov radiation [12].

Results

Amplification of target nucleic acids from nasopharyngeal swab specimens spiked with virus

Amplification of nucleic acid extracted from each of 15 NP specimens spiked with influenza A virus was attempted with *Taq* DNA polymerase-based and *Tth* enzyme-based PCR. Products of the expected size (311 bp) and giving equivalent band intensities were

obtained for four specimens with both polymerases. Viral nucleic acids from six specimens were detected with at least 10-fold more sensitivity when amplified by *Tth* enzyme than by *Taq* DNA polymerase (e.g., nos. 1 and 6 in Fig. 1). For five specimens, no products were detected with *Taq* polymerase (e.g., specimens 2–5, Fig. 1). Positive control RNA was amplified equally by *Taq* and *Tth* DNA polymerases (not shown). The data indicated that, for some NPs, PCR by *Taq* polymerase was partially or totally inhibited as compared with that by the *Tth* enzyme-based method.

Effect of mixing inhibitory or partially inhibitory extracts with control RNA in PCR

A sample from each of the five-fold dilutions of an inhibitory sample extract was mixed with the control RNA (isolated from H₂O spiked with virus suspension), cDNA was made and was then amplified by *Taq* polymerase. The addition of 1 μ l of extract from spiked specimen nos. 2 and 5 resulted in no products from control RNA. The amplification was restored when the extract from specimen no. 2 was diluted at least 1 in 25 and that from specimen 5 was diluted at least 1 in 5. To eliminate inhibition from other NP specimens, dilutions between 1 in 5 and 1 in 125 were required (not shown).

Effect of VIB and calcium alginate swab on PCR with *Taq* polymerase

The expected 311-bp amplified DNA fragment was detected in virus diluted to 1 in 10³ in water, in VIB and in H₂O incubated with calcium alginate swabs. Identical results were obtained when different batches of VIB and calcium alginate swabs were examined.

Fig. 1. Example of influenza A virus PCR inhibition by NP specimens. **A**, PCR with *Taq* DNA polymerase; **B**, PCR with *Tth* DNA polymerase. The dilutions of virus used for spiking each NP specimen were: **a**, 10²; **b**, 10³. **M**, molecular size marker (100 bp, DNA ladder, Life Technologies).

Effect of inhibitors from NP specimens in RT and PCR

To characterise the inhibitor in NP specimens with respect to its effect in the reverse transcription step, control RNA was reverse transcribed in the presence of α - 32 P-dCTP, with and without additional nucleic acid (RNA) from a representative inhibitory NP specimen (no. 5) spiked with the virus. The incorporation of 32 P-labelled dCTP in the cDNA synthesised by reverse transcriptase decreased with the increased concentration of the added nucleic acids extract (Fig. 2). To examine the effect of inhibitors on the PCR step, cDNA was synthesised from control template RNA and different volumes of inhibitory extract from virus-spiked NP specimen no. 5 were added before amplification with *Taq* DNA polymerase. The yield of amplified product decreased with the increasing addition of inhibitory extract. The addition of 4 μ l of nucleic acid extract completely inhibited PCR amplification by *Taq* DNA polymerase. Qualitatively similar results were obtained for both RT and PCR steps with other inhibitory or partially inhibitory extracts (not shown). These results indicated that both the reverse transcription and PCR steps were adversely affected by the inhibitors in NP specimens.

Discussion

In this laboratory it had been observed that, for some influenza A virus culture-positive NP specimens, cDNA synthesis followed by PCR with *Taq* DNA polymerase gave false negative results, whereas amplification in a single tube [11] with *Tth* DNA polymerase gave positive results, as expected. In the present study, PCR amplification of standardised amounts of influenza A virus in NPs by *Taq* DNA polymerase and *Tth* DNA

polymerase were compared. Amplification of 10-fold dilutions of virus in NP specimens showed that *Tth* enzyme detected 10-fold fewer copies than *Taq* polymerase for several of the NP specimens studied. When control RNA was mixed with inhibitory extracts from virus-spiked NP specimens and subjected to PCR with *Taq* polymerase, the extent of inhibition was shown to vary between specimens. The possibility that differences in the amount of inhibitors found in different NPs were due to differences in patients' medical conditions or stages of infection needs to be investigated further.

The results of the present study showed that the calcium alginate swabs and VIB transport medium did not inhibit PCR. Similarly, He *et al.* [13] successfully used calcium alginate or cotton NP swabs in PCR-based assays. In contrast, Wadowski *et al.* [14] found that calcium alginate and the aluminium shaft component of the swab in NP specimens were inhibitory for PCR. These differences in results may have been caused by differences in target nucleic acid preparation methods.

The fact that the activity of both reverse transcriptase and *Taq* DNA polymerase was inhibited by extracts from some NP specimens suggests that amplification by *Taq* DNA polymerase of target nucleic acids from other common respiratory infectious agents in an NP specimen may be inhibited. Unpublished experiments in this laboratory with *Mycoplasma pneumoniae* and respiratory syncytial virus in NP specimens showed inhibition of amplification by *Taq* DNA polymerase compared with that by *Tth* DNA polymerase, supporting further the notion that the PCR inhibition occurred by directly inhibiting the enzymic activity and that the inhibition was independent of the target or primer set used.

Fig. 2. Incorporation of α - 32 P-dCTP in the cDNA synthesised by reverse transcriptase from: a, control RNA alone or; b, control RNA plus 1 μ l of inhibitory extract from spiked NP specimen no. 5 diluted 1 in 625; c, 1 in 125; d, 1 in 25; e, 1 in 5; f, undiluted.

As amplification by *Tth* enzyme was detected in all samples, the lack of detectable products from samples nos. 2–5 by *Taq* DNA polymerase-based PCR was not due to the absence of target RNA in the samples. In the PCR, reduced product DNA synthesis sometimes may occur because of the presence of nuclease activity in the nucleic acid sample. As samples that were inhibitory to amplification by *Taq* polymerase were amplified by *Tth* enzyme, the inhibition was not due to the presence of nucleases. The presence of unwanted metal ions in the nucleic acid sample could also inhibit PCR amplification. In a preliminary experiment, with nucleic acid isolated with Chelex-100 (BioRad) to remove metal ions [15] from inhibitory samples, no improved amplification by *Taq* DNA polymerase was detected (unpublished observation). This suggests that the inhibition was not caused by metal ions. Potent inhibitory proteins such as haemoglobin or carbonic anhydrase have been reported by other investigators to be simultaneously released with nucleic acid even when the standard phenol-

chloroform-isoamylalcohol extraction method is used [16]. The QIamp procedure used in the present study allows rapid purification of nucleic acids from biological samples suitable for PCR-based assays [17]. However, it remains to be determined whether some specimens used in the present study contained large amounts of inhibitors that were not all removed and hence may have been co-purified to some extent with RNA by the QIamp procedure, causing inhibition.

Reports on the successful use of *Tth* DNA polymerase-based RT-PCR protocol incorporating UNG sterilisation in a single tube reaction are limited [7, 11]. In the present study, amplification of target nucleic acid by *Tth* enzyme with such a single tube reaction protocol was found not to be perturbed by the inhibitors of *Taq* DNA polymerase present in the NP specimens. The single tube reaction protocol also reduces the sample handling time and the chances of exogenous contamination. In routine diagnostic protocols, these advantages make the presently used *Tth* enzyme-based PCR method preferable to cDNA synthesis followed by amplification with *Taq* DNA polymerase.

In summary, the present study showed that some NP specimens may contain inhibitors of both reverse transcriptase and *Taq* DNA polymerase, and the inhibitors may co-purify with nucleic acid. Dilution of nucleic acid extracts from such specimens (which would also decrease the sensitivity of the PCR), or the use of *Tth* DNA polymerase instead of *Taq* DNA polymerase, can minimise the possibility of false negative PCR amplification.

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