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Optimized PCR Amplification of Influenza A Virus RNA Using Tth DNA Polymerase, Incorporating Uracil N Glycosylase (UNG) in a Single Tube Reaction

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An optimized reaction condition for amplification of influenza A virus RNA, by thermus thermophilus (Tth) DNA polymerase-based PCR, incorporating uracil N glycosylase (UNG) and dUTP in the reaction has been determined. dUTP could not be substituted for all dTTP sites when UNG was present in the reaction. The relative concentration of dUTP and dTTP has been optimized for allowing amplification of the target RNA. It has been verified that the amplified product DNA had sufficient dUTP and was digestable by UNG. Using the optimized reaction condition, influenza A virus-specific DNA fragment could be amplified and detected in 15 of 15 culture positive (for influenza A virus) nasopharyngeal specimens. J. Clin. Lab. Anal. 11:323–327, 1997. © 1997 Wiley-Liss, Inc.

Key words: RT-PCR; uracil N glycosylase; thermus thermophilus (Tth); DNA polymerase

INTRODUCTION:

The polymerase chain reaction (PCR) allows a thermostable DNA polymerase to amplify a few copies of the target nucleic acids (DNA or RNA) greater than a millionfold. In view of its enormous sensitivity, the PCR technique has had widespread application in nucleic acid amplification-based diagnostics (1–3). The amplification of a RNA target such as the genome of influenza A virus involves conversion of RNA to copy DNA (cDNA) by reverse transcriptase (4). Subsequently the cDNA is amplified by a thermostable DNA polymerase. Both reverse transcriptase and DNA polymerase activity is present in Tth DNA polymerase (5). The amplification of RNA is therefore possible by Tth DNA polymerase alone in a single reaction procedure that facilitates diagnostic assay by PCR (6).

An unwanted drawback in a PCR is the fact that even a trace quantity of exogenous carryover template contaminant can often lead to false positive amplified product. Carryover template amplification is preventable if amplifications are performed such that every dTTP in the amplified DNA is substituted by dUTP. Under this condition, carryover contaminant from a previous PCR amplified product containing dUTP, but not the specific native target nucleic acid, becomes susceptible to degradation by the enzyme uracil N-glycosylase (UNG). Therefore, if the PCR reaction mixture is treated with UNG prior to amplification, the carryover contaminant will not reamplify (7). Thus a PCR condition, with a UNG digestion step at the precycle, that allows sufficient dUTP incorporation in the amplified product DNA so that it can be digested by UNG is of enormous importance for application in routine PCR-based clinical diagnostic protocols.

The efficiency of incorporation of dUTP by different DNA polymerases is different and the incorporation by the Tth DNA polymerase is extremely inefficient (8). The efficiency of incorporation of dUTP by a thermostable DNA polymerase also depends on the percentage of riboadenylate (rA) in the template. Increased concentration of dUTP is required in the reaction for efficient reverse transcription of templates with a high percentage of rA residues (9). In addition, in Tth enzyme-based amplification of RNA in a single tube reaction, the UNG residual activity (after the carryover nucleic acid digestion) can degrade the nascent dUTP incorporated cDNA at the reverse transcription step and can lead to little or no amplification. Possibly due to all these problems, reports on the Tth DNA polymerase-based RT-PCR where UNG and dUTP have been successfully incorporated are limited (2). The present report is on the optimization of such a RT-PCR protocol (UNG incorporated) for amplification and detection of influenza A virus. Optimization of the dNTPs and dUTP concentration in the reaction and the parameters (temperature and duration of incubation) for precycle and cycle condition based on the template and primer combination is an absolute requirement for reproducible level of amplified prod-

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uct. In the present communication, dUTP and dTTP concentrations in the reaction mixture have been optimized so that there is efficient incorporation of nucleotides, and the reverse transcription (RT)-PCR by Tth polymerase escapes the effect of residual or renatured UNG activity. The optimized Tth enzyme-based PCR reaction, has been applied for detection of influenza A virus in a set of culture positive (for influenza A virus) nasopharyngeal (NP) clinical specimens.

MATERIALS AND METHODS

Virus Strain and Nucleic Acids

Control influenza A virus (H1N1) stock was obtained from the Department of Public Health, San Diego. The titer of the stock virus was 4×10^4 $TCID_{50}$/ml. Viral nucleic acid (RNA) was purified from 200 µl of either serial (1:10) diluted virus stock or undiluted culture positive (for influenza A virus) nasopharyngeal specimens (obtained from Virology Laboratory, UCSD Medical Center) using the QIamp nucleic acid isolation kit (Qiagen, Chatsworth, CA) following the manufacturer's suggested protocol. The purified RNA was eluted in 100 µl of 10 mM Tris-HCl pH 9. PAW109 RNA was obtained from Perkin Elmer, Cetus (Norwalk, CT) and was used as negative control template for PCR.

Amplification by Tth DNA Polymerase

The sense (5′ CCGAGATCGCAGCA GAG ACTTG AAGAT 3′) and antisense (5′ GGCA AGTGCACCAG-CAGAATAACT 3′) primers derived from the conserved region of the matrix gene of influenza A virus were identical to those used in a previously reported study (1). Uracil N glycosylase, Tth DNA polymerase, dATP, dGTP, dCTP, dTTP, and dUTP were all purchased from Perkin Elmer, Cetus. The reverse transcription and PCR amplification was performed by Tth enzyme in bicine buffer (Perkin Elmer) and Mn $(OAc)_2$. The concentration of dATP, dGTP, dCTP each in all the reaction was 300 µM; that of dTTP and dUTP was as required in each specific experiment. In a 50-µl reaction mixture, 0.5 U of UNG, 2.5 U of Tth enzyme, 10 µl of RNA template, and 0.5 µM each of sense and antisense primers were used. For a total dNTP concentration of 1.2 mM, $Mn(OAc)_2$ was 2.5 mM. For every 100 µM change (increase or decrease) of dUTP and/or dTTP, $Mn(OAc)_2$ concentration in the reaction was changed accordingly by 100 µM. The final volume of the reaction was made 50 µl by adding the required volume of DEPC treated H_2O.

RT-PCR was performed in a single reaction mixture in a Perkin Elmer thermocycler 2400. Precycle condition consisted of 10 min at 25°C for digestion by UNG, 2 min at 95°C for RNA secondary structure removal and inactivation of UNG, then 30 min at 60°C for reverse transcription by Tth DNA polymerase. Amplification was allowed in 40 cycles of reaction. Each cycle consisted of 10 sec denaturation at 94°C, 15 sec primer annealing at 60°C, and 30 sec primer extension at 72°C. The final reaction product was brought to 72°C and was held at that temperature until used for analysis by agarose gel electrophoresis. Ten µl of the product were analysed by electrophoresis in a 2% agarose (Marine colloid, Maine) gel containing 0.5 µg/ml ethidium bromide. The electrophoresis was performed in 1× TBE (89 mM Tris-borate, 2 mM EDTA, pH 8.3) buffer. The DNA bands in the gel were visualized through an UV transilluminator.

Hybridization in Liquid and Autoradiography

Hybridization in liquid was conducted following previously described method (10). In brief, 10 µl of the amplified DNA was mixed with 10 µl ^{32}P labeled oligonucleotide probe (5′ CCTAAGTTTTC TATACAGTTTAACT 3′; BRL) complementary to the internal sequence, in 2× liquid hybridization buffer (0.015 M NaCl, 0.01 M Disodium EDTA, pH 8.0). The probe was labeled by phosphorylation at the 5′ end with ^{32}P labeled ATP and T4 polynucleotide kinase (from Gibco, BRL, Gaithersburg, MD) following a standard protocol (11). The 20 µl mixture was heated for 5 min at 95°C and then hybridization was allowed by incubating the mixture at 58°C for 10 min. The product DNA hybridized with the probe was separated from the unhybridized probe by electrophoresis in a nondenaturing 6% polyacrylamide gel in TBE buffer. Ten µl of each hybridized samples were used for such electrophoretic separation. The gel was dried and exposed to Kodak X-Omat X ray film for 12–16 hours. The film was then developed and the autoradiographic bands analysed.

RESULTS

Optimization of dUTP Concentration

Using RNA purified from diluted (1:10) control influenza A virus stock, reverse transcription (RT)-PCR amplification by Tth DNA polymerase was examined at varied concentrations of dUTP keeping other dNTPs (dATP, dCTP, dGTP) fixed at 300 µM. There was no UNG in the reaction mixture, and the reaction did not undergo any precycle step for digestion by UNG. Figure 1 shows the analysis of the amplified products. The expected 311 bp DNA fragment was amplified when all four dNTP concentrations were 300 µM and no dUTP was added (Fig. 1, lane 1), or when dUTP concentration in the reaction was in the range of 300–600 µM (Fig. 1, lanes 5–8). At dUTP concentration equal to 700 µM and higher, there was considerable synthesis of smaller fragments that generated smeared DNA band patterns. The relative yield of specific product DNA fragment was highest between 400–600 µM concentration of dUTP; 500 µM was taken as the best workable dUTP concentration for the template and primers combination used in the experiments. However, when UNG was added to the reaction mixture, very little or no synthesis of specific DNA fragment was detected, using either 400, 500,

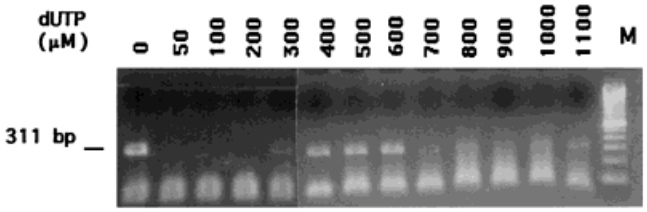

Fig. 1. RT-PCR amplification of control RNA purified from a stock Influenza A virus (H1N1), by Tth DNA polymerase replacing dTTP with varied concentration of dUTP in the reaction. There was no UNG in the reactions. Lane M: 100 bp ladder molecular size standards (BRL).

or 600 μM of dUTP in combination with 300 μM of other dNTPs (dATP, dCTP, dGTP) in the reaction (for 500 μM dUTP see Fig. 2A lane 1; others not shown).

Optimization of dUTP Relative to dTTP

The concentrations of dUTP and dTTP, keeping other dNTPs (dATP, dGTP, dCTP) in the reaction at 300 μM, were adjusted to escape dUTP substitution for dTTP in the entire segment (to be amplified) of interest or at least at the more UNG susceptible sites in the cDNA sequence. Therefore, first it was examined whether or not it is possible to adjust the concentration ratio of dUTP to dTTP to allow amplification of the specific DNA fragment while including UNG in the reaction. Figure 2A shows agarose gel analysis of the product where dTTP concentration was varied from 0–300 μM keeping dUTP concentration fixed at 500 μM and other dNTPs (dATP, dGTP, dCTP) each at 300 μM. The reaction was performed in the presence of 0.5 U of UNG, and the reaction underwent a precycle step for digestion by UNG. Amplification was obtained for the control mixture where 300 μM each of all four dNTPs were present (Fig. 2B, lane C). No amplified product was detected when dTTP concentration was below 75 μM (see lanes 1–4). The 311 bp amplified DNA fragment was generated when dTTP concentration was between 75–300 μM (lanes 4–7). Figure 2B shows the analysis of the reaction product when all four dNTP concentrations were fixed at 300 μM, and dUTP concentration was varied from 0–500 μM. The 311 bp fragment was found to be generated more or less at the same level when the dUTP concentration ranged 100–500 μM (Fig. 2B, lanes 1–5). Thus the specific product DNA fragment could be generated when dUTP (μM) : dTTP (μM) were 500 : 100; 500 : 200; 500 : 300 (Fig. 2A) and 100 : 300; 200 : 300; 400 : 300; and 500 : 300 (Fig. 2B) in the presence of UNG in the reaction.

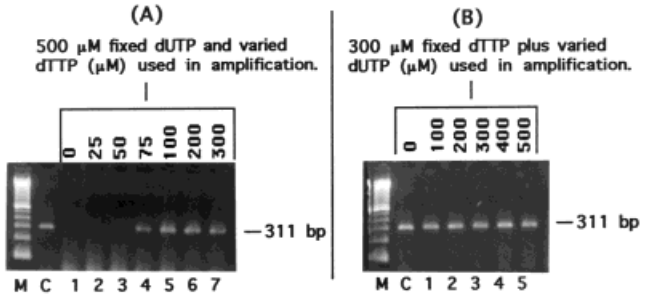

Fig. 2. In presence of UNG, RT-PCR by Tth DNA polymerase when dUTP concentration was kept fixed at 500 μM and dTTP concentration was varied as indicated on top of the lanes (Panel **A**) or dTTP concentration was kept fixed at 300 μM and dUTP concentration was varied as indicated on top of each lane (Panel **B**). The concentration of other dNTPs (dATP, dGTP, dCTP) was each 300 μM, and 0.5 U of UNG was used per each 50 μl reaction. Lane M: 100 bp ladder molecular size standard.

Reamplification of an Aliquot of the Product

To test whether or not the amplified DNA fragment generated using the above combinations of dUTP : dTTP had dUTP sites that will also be a substrate for UNG digestion, the following experiment was conducted. About 5% of each of the amplified product from reactions described in Figure 2 was digested with 0.5 U of UNG in the precycle and then amplified with Taq DNA polymerase using 200 μM each of four dNTPs in the reaction. Out of 50 μl reaction, 10 μl was analysed by electrophoresis in an agarose gel (Fig. 3). No amplified product was detected corresponding to the aliquots of product generated originally using dUTP : dTTP of 300 : 300, 400: 300, 500 : 100; 500 : 200 and 500 : 300 (lanes 6 & 10).

Whereas control amplification of the aliquot from the product generated with only 300 μM, each of all four dNTPs (described in Fig. 2A, B, lanes marked C) was obtained as expected (lanes 1 and 7). Product generated with dUTP : dTTP

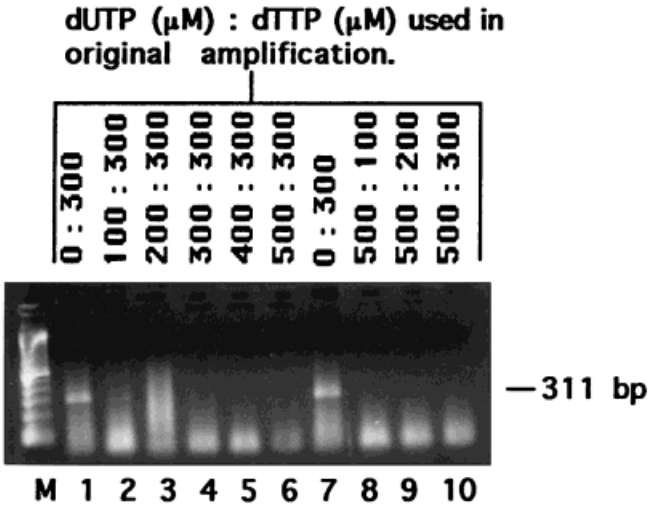

Fig. 3. Digestion by UNG and subsequent reamplification of an aliquot of the previously amplified product obtained using different combination of dUTP : dTTP concentrations. The μM concentration of dUTP : dTTP used originally to generate the amplified product DNA is shown on top of each lane; 50 μl reaction mixture contained 200 μM each of dATP, dGTP, dCTP, dTTP, 1.25 U Taq DNA polymerase (Perkin Elmer), 0.5 U UNG, 0.5 μM each primers and 2.5 μl of the aliquot. Lane M: 100 bp molecular size standards.

of 100 : 300, however, when reamplified after digestion with UNG produced DNA bands that were detectable (lane 2). There was some smeared band pattern ~ 300 bp size range when the aliquot was from product generated with 200 : 300 of dUTP : dTTP (lane 3). Thus, dUTP:dTTP concentration of 100:300, or 200:300, although allowing amplification, cannot be used in a protocol for prevention of carryover contaminant. However, 500 : 100, 500 : 200, 500 : 300, 400 : 300, or 300 : 300 of dUTP : dTTP will allow amplification in the presence of UNG, and the product also would be digestable by UNG. Although any of these concentration ratios will work, a median 500 : 300 of dUTP : dTTP was assumed as the best optimized concentration that will incorporate sufficient dUTP at the sites of dTTP without adversely effecting amplification.

Amplification and Detection of the Virus in Clinical Specimens

Using this optimized reaction condition, nucleic acid from 15 nasopharyngeal specimens (culture positive for influenza A virus), were PCR amplified for detection. Figure 4A shows the analysis of the product by agarose gel electrophoresis for RNA from eight representative culture positive NPs and two control RNA samples. DEPC treated H_2O and a nonspecific target PAW 109 RNA were used as the negative controls for amplification (not shown). For enhanced sensitivity of detection, the PCR product is generally hybridized with a labeled oligonucleotide probe complementary to an internal sequence. Hybridization in liquid (10) being a rapid procedure has been applied in this study.

The result of autoradiography of the hybridized sample is depicted in Figure 4B. In autoradiographic analysis, two specific hybridized bands were observed consistently. Mobility of DNA in a nondenaturing polyacrylamide gel being extremely sensitive to its (the migrating DNA molecule) conformation, one of the bands was for target strand hybridized with the probe, and the other one was possibly due to partial strand displaced hybridized molecule. The sensitivity of the assay was delineated by analysing amplified RNA extracted from 1:10 serial diluted control virus specimen. Amplifications of RNA from 10^{-3}- and 10^{-2}-fold diluted control virus are shown in Figure 4, lane 1 and 2, respectively. The minimal titers of the virus for which the appropriate DNA band or the probe hybridized signal band was visualized by electrophoresis and autoradiography were ~ 10 to 1 and < 1 $TCID_{50}$ s, respectively, in the tested volume (10 µl) of the sample. Influenza A virus was detected in all clinical specimens except two, by agarose gel analysis (Fig. 4A, lanes 3–10). The virus could, however, be detected in all 15 specimens by hybridization with the labeled probe and autoradiography (as shown for representative specimens in Fig. 4B).

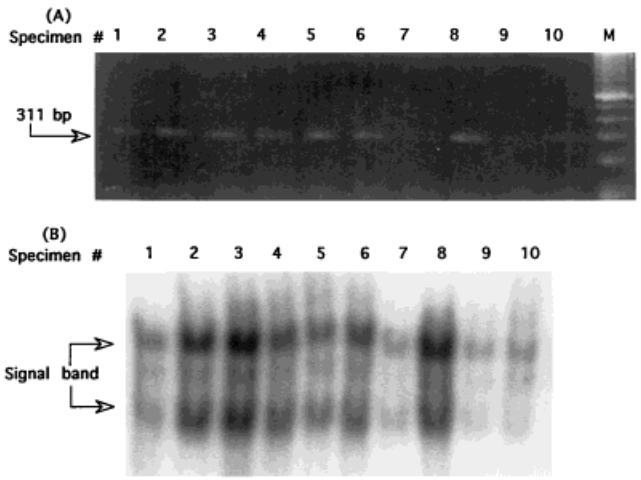

Fig. 4. Amplification of nucleic acid (RNA) purified from representative influenza A virus culture positive nasopharyngeal specimens using the optimized concentration of dUTP and dTTP in presence of UNG in the reaction. Panel **A**: Analysis of the product by agarose gel electrophoresis. Panel **B**: Autoradiographic analysis of the product after hybridization in liquid. Lanes 1–2, positive control RNA isolated from 10^{-3}- and 10^{-2}-fold diluted virus. Lanes 3–10, RNA isolated from influenza A virus culture positive specimens.

DISCUSSION

The amplification of specific DNA fragment has been found to be extremely sensitive to the dUTP concentration in the reaction. At dUTP concentration < 300 µM, no amplification was detected (Fig. 1, lanes 2–4). At dUTP concentration of 700 µM or higher, smeared DNA band patterns were observed. This was possibly due to products generated by nonspecific priming and/or premature termination of DNA polymerase in the presence of increased metal ion (Mn++) concentration in the reaction (5). In the presence of UNG, there was no synthesis of the expected 311 bp DNA fragment using even the concentration (500 µM) of dUTP that was found optimum for highest yield of specific product DNA in the reaction (Fig. 2A, lane 1). This might be due to the fact that residual and/or reactivated UNG was digesting away the newly transcribed cDNA during reverse transcription step at 60°C. In fact, even after 30–40 cycles of varied temperature ranging from 55°C to 95°C, reactions having dUTP and UNG are recommended to be stored at 72°C. This is because UNG is sufficiently reactivated when the reaction is brought at lower temperature and degrade the amplified dUTP containing product DNA (12).

There are no reported data on the relative level of reactivated UNG at 60°C, especially when incubation is as long as 30 minutes. It is quite possible that inactivation for 2 minutes at 95°C was not sufficient, and/or the reactivation at 60°C might be enough to cause degradation at a few sites of the cDNA generated by Tth DNA polymerase. Also it may be that some sites of the cDNA where dUTP substituted for dTTP become more susceptible to UNG digestion than others. Thus even with very little activity, UNG could digest at those sites of nascent cDNA and cause minimal or no amplification. This problem has been addressed by optimizing the concentration

of dUTP relative to that of dTTP in the reaction (Fig. 2A,B). The experimental result (shown in Fig. 2A,B) has demonstrated that several combinations of dUTP and dTTP concentration in the reaction will produce amplified DNA product. However, certain combinations of dUTP and dTTP concentration may still result in PCR products that are not fully degraded by the UNG digestion (Fig. 3).

Tth DNA polymerase maintains > 75% reverse transcription activity between 55°C and 70°C (9). If reverse transcription is allowed at the high end of this temperature range, the UNG may have little or no effect (12). However, when the lower end of the temperature range has to be used for reverse transcription, the effect of UNG on dUTP incorporated cDNA may be significant. Therefore, the present optimization procedure would be of particular importance when primers of relatively low melting temperature need to be used. In the previously published RT-PCR protocol for HIV, reverse transcription was performed at temperatures as high as 70°C; yet dTTP was used in conjunction with dUTP in the reaction (2). It is not known whether such addition of dTTP and dUTP together was an essential requirement for the specific template RNA (HIV RNA), and the primer set combination or the complete substitution of dUTP for dTTP was also possible. The experimental data in the present report provided the rationale for selecting a combination of dUTP and dTTP concentration in the reaction for incorporating UNG in Tth polymerase based RT-PCR, using matrix protein gene of influenza A virus as the template for amplification. The optimized reaction condition was further validated by the detection of the virus in culture positive specimens (Fig. 4).

In conclusion, using RNA of influenza A virus, a specific segment of a virus specific gene could be amplified based on reverse transcription and PCR by Tth DNA polymerase in a single tube reaction. Uracil N Glycosylase and dUTP was incorporated in the reaction for carryover digestion. The concentration of dUTP to dTTP was optimized to 500 μM : 300 μM for minimizing any nascent cDNA digestion by residual and reactivated UNG at 60°C reverse transcription step. PCR using this reaction condition allowed detection of all the culture positive influenza A virus nasopharyngeal samples examined in this report.

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