Evaluation of PCR Assays in Presence of Antibody to Thermostable DNA Polymerases for Detection of Microbial Agents: Avoiding False Negative Results for Specimen Containing Low-Titer Agent

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The serial low-titer specimens of Influenza A virus and Adeno virus type 7 were tested for the presence of virus specific genes by PCR based on Tth DNA polymerase and by that based on Taq DNA polymerase, in the absence and presence of antibody to the respective DNA polymerases. Increased product DNA synthesis and higher sensitivity of detection were observed in the pres-

ence of antibody compared to those in the absence of antibody. 10- to 100- fold lower titer specimen of Influenza A virus and 10- fold lower titer specimen of Adeno virus could be detected in the presence of antibody than those detected in the absence of antibody to the appropriate DNA polymerase, in a PCR. *J. Clin. Lab. Anal.* 12:238–241, 1998. © 1998 Wiley-Liss, Inc.

Key words: Taq DNA polymerase; Tth DNA polymerase; monoclonal antibody

INTRODUCTION

The application of polymerase chain reaction (PCR) methodology has brought about a revolutionary change in the field of clinical diagnostics, simply because it allows a rapid and convenient million-fold amplification of a few copies of the target nucleic acid (1). Qualitative and quantitative PCR results are applied in pathogen identification (2–5), determining a pathogen load in a patient and following up the susceptibility of the pathogen to an appropriate drug (6). However false positives and false negatives in PCR amplification are observed quite often. Such false PCR results can create confusion in the diagnosis of the causative agent and can have serious consequences in the therapeutic decision relating to the application and continuation or withholding of proper antibiotic and antiviral drugs.

False positives can result from exogenous nucleic acid contaminant in the PCR reaction mixture. Several authors identified the sources of false positives and determined methods of avoiding and interpreting them in a PCR amplification (7, 8). A negative PCR that results due to the inhibitors have been reported in many studies with clinical specimens and such false negatives are avoidable either by the use of a procedure that can purify the nucleic acid adequately, or by using inhibitor-insensitive DNA polymerase (9–11) in the reaction. So far, little attention has been paid to avoid the negative false PCR with clinical specimens that may result from the intrinsic limiting condition of the reaction, especially when the target nucleic acid is available in a very low quantity or copy

number. Nonspecific priming at lower temperatures and the corresponding nonspecific PCR products (such as primer dimer formation) are the major artifacts that can limit the yield of product synthesis and sensitivity of a PCR. These artifacts are more prominent when the target nucleic acid is relatively low, and can compete with the specific PCR product.

The use of “Hot start” PCR has been reported for avoiding the PCR artifacts and for enhancing the sensitivity and specificity of amplification (12). The Hot start PCR procedure, however, is time consuming and inconvenient, requiring the additional step of bringing the reaction to high temperature before the enzyme is added. This brings the reaction to the risk of cross-contamination and thus the procedure is unsuitable for application in routine diagnostic protocols. The monoclonal antibody to DNA polymerase is also known to reduce the PCR artifacts and increase the sensitivity of the reaction (13). Since the monoclonal antibody to DNA polymerase can be added straight to the PCR reaction mixture, use of antibody-to-DNA polymerase could be an effective step for avoiding false negative PCR amplification when the microbial agent in question is expected to be present in low titer in a specimen. This article evaluates the yield of specific PCR product and the sensitivity of assay using monoclonal

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antibody against Taq DNA polymerase and monoclonal antibody against Tth DNA polymerase when amplification was performed by Taq and Tth DNA polymerase respectively. Two different microbial agents were used as targets for evaluation of the assays based on PCR in presence of antibody to the DNA polymerase in the reaction.

MATERIALS AND METHODS

Microbial Specimens

Influenza A (H1N1) virus (Titer 4×10^4 TCID₅₀/ml), and Adenovirus type 7 (Titer 1×10^5 TCID₅₀/ml), were obtained from the Department of Public Health Laboratory, San Diego. The viruses were originally isolated from patient's specimens, propagated, and the stock of each virus was prepared and titered by the department of Public Health Laboratory, San Diego. Each microbial stock was serially diluted 10-fold in the veal infusion broth (VIB) transport medium, and nucleic acid was isolated from diluted specimens using the QIamp kit (Qiagen, Inc), following the procedure provided by the manufacturer.

PCR by Taq DNA Polymerase and Tth DNA Polymerase in the Absence and Presence of Respective Antibody

The sense and antisense primers and PCR reaction condition for Influenza A virus assay by Tth DNA polymerase and that by Taq DNA polymerase were identical to those described in previous publications (14, 11). The primers for Adeno virus assay were the same as those used in another study (15). The 50 μ l PCR reaction mixture for Adeno virus contained 10 μ l target nucleic acid (DNA), 0.5 μ M each of sense and antisense primers, 0.5 U of Uracil N glycosylase (UNG), 200 μ M each of four dNTPs, and either the combination of 1.25 U of Taq DNA polymerase (Perkin Elmer), 2.5 mM MgCl₂ in 1X PCR buffer (10 mM Tris, pH 8.3 50 mM KCl; Perkin Elmer) for Taq DNA polymerase or the combination of 1.25 U Tth DNA polymerase, 2.5 mM MgCl₂ in the adjusted buffer (10 mM Tris, pH 8.3; 100 mM KCl) for Tth DNA polymerase. Precycle conditions consisted of incubation at 20°C for 10 min for UNG digestion of any carryover contaminants, and a second incubation at 95°C for 5 min for UNG inactivation. Amplification was performed using 40 cycles, each cycle consisting of 94°C for 10 sec and 68°C for 1 min (for annealing and primer extension). One cycle of post-amplification consisted of 7 min at 72°C and 10 min at 95°C. The reaction product was finally stored at 72°C and analysed within one hour. Monoclonal antibody to Taq and that to Tth DNA polymerases (TaqStart and TthStart) were purchased from Clontech (Palo Alto, CA). The stock antibodies were diluted and added to the reaction mixture following the suggested protocol provided by the supplier. All reactions were allowed in MicroAmp tubes (Perkin Elmer) in the thermocycler 2400 (Perkin Elmer).

Analysis of Product DNA by Agarose Gel Electrophoresis

Out of each 50 μ l reaction product, 10 μ l were analyzed by electrophoresis in a 2% agarose gel containing ethidium bromide. The electrophoresis was conducted in a TBE (89 mM Tris-borate, 2mM EDTA, pH. 8.3) buffer. The DNA bands were visualized through an UV transilluminator.

RESULTS

The analysis of product DNA of Tth DNA polymerase based RT-PCR in the absence and presence of antibody to Tth enzyme for Influenza A virus specimens is shown in Figure 1a. In the absence of antibody, nucleic acid isolated from sample diluted upto 10^{-3} was detectable whereas that from 10^{-5} diluted sample was distinctly detected when antibody was present in the reaction. (Compare panel A with panel B.) Thus the presence of antibody increased the sensitivity of assay 100-fold. However, in PCR with Taq DNA polymerase, presence of antibody increased the sensitivity 10- to 50-fold (not shown). Figure 1b shows analysis of product from PCR by Taq DNA polymerase for Adenovirus specimen, without and with antibody to Taq DNA polymerase in the reaction. In presence of antibody a sharp 10-fold increase in sensitivity of detection was observed. (Compare panel A with panel B.) When PCR was allowed by Tth DNA polymerase for Adeno Virus, a similar 10-fold increased sensitivity was also observed in presence of antibody compared to that in the absence (not shown). The formation of primer dimer, one of the causes of limiting the sensitivity of a PCR, was observed in reactions with nucleic acid isolated from 10^{-5} or more diluted samples when no antibody was added, whereas primer dimer formation was consistently absent in all reactions in the presence of Taq antibody (Fig. 1b). Increased product DNA synthesis was detected when antibody was used in the reaction for both viruses at each dilution as compared to that in the absence of antibody (Fig. 1a and Fig. 1b). Qualitatively similar increased DNA synthesis and sensitivity of detection were obtained in PCR assays of other microbial agents (in an unpublished observation in our laboratory) such as Mycoplasma pneumonia and Chlamydia pneumonia in presence of appropriate antibody to the DNA polymerase used (Taq or Tth DNA polymerase) compared to those in the absence of antibody.

DISCUSSION

The results of this study suggest that a microbial agent in a specimen having titer 10- to 100-fold less than what is detectable by PCR, based either on Taq or Tth DNA polymerase alone, will be detected when antibody to the appropriate DNA polymerase is used in the reaction. In contrast, other investigators have found either negative or marginal influence of antibody (16) using GeneAmp tube as the reaction container for PCR. However, they obtained increased synthesis of prod-

Fig. 1. Analysis of PCR product for Influenza A virus specimens by Tth DNA polymerase (a) and that by Taq DNA polymerase for Adeno virus (b) in the absence (panel A) and presence (panel B) of monoclonal

antibody to the respective DNA polymerases. Dilutions of the specimen are as indicated on top of each lane. Lane M: Molecular size marker 100 bp DNA ladder.

uct DNA in presence of antibody, using silicon-glass chip reaction containers, and the result was in qualitative agreement with that in the present report. Specific DNA oligomers termed “aptamers” that bind reversibly (as the temperature is changed) with the Taq DNA polymerase and that mimic the effect of the antibody, have been isolated (17) and thus aptamers could be an alternative reagent to antibody against Taq DNA polymerase.

Specimens used in this report were prepared by spiking the microbial agent in a medium. This allowed an evaluation of the effect of the antibody for samples having controlled low-titer agents in control medium. A recent report indicated that false negative PCR by Taq DNA polymerase may result due to the presence of inhibitors in certain clinical specimens, and such false negative results could be avoided by using Tth DNA polymerase based PCR (11). Thus, although the use of antibody in either Taq or Tth enzyme-based PCR will increase

sensitivity, the Tth enzyme-based PCR in presence of antibody will provide protection against inhibitors in the specimen, and improve the sensitivity of detection as well, for the microbial agent in question in a specimen.

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