Rapid Detection of *Bordetella pertussis* by Real-Time PCR Using SYBR Green I and a LightCycler Instrument

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A polymerase chain reaction (PCR) assay in real-time for detection of *B. pertussis* using SYBR green I as the reporter fluorophore and LightCycler instrument (a thermocycler coupled to a fluorescence detection device) was established and evaluated. The amplified amplicon using series diluted control prototype strain (ATCC strain #9797) of *B. pertussis* was analyzed for the fluorescent melting profile, and melting temperature (T_m) was determined. When examined, amplicons

using a representative set of clinical isolates of *B. pertussis* were found to have the same T_m value ($86 \pm 0.5^\circ\text{C}$, the specificity parameter of detection) as the control prototype strain as expected. Amplified product was also analyzed and detected by agarose gel electrophoresis. The detection limit by fluorescent profile and T_m analysis was 10-fold better than that detected by agarose gel analysis. J. Clin. Lab. Anal. 18:265–270, 2004. © 2004 Wiley-Liss, Inc.

Key words: *B. pertussis*; real-time PCR; melting temperature (T_m); LightCycler

INTRODUCTION

Pertussis (whooping cough) is a well-known childhood respiratory disease and is caused primarily by *Bordetella pertussis* infection (1). Although immunization programs brought the disease under control for decades, studies conducted in recent years indicate that the incidence rate of pertussis is on the rise among unvaccinated children (2), and is reemerging as a potential threat even among those who are vaccinated (3,4). The disease is now quite prevalent among adults and adolescents in whom the disease symptoms are often atypical (5,6). They are continuing to be a source of *B. pertussis*, and this is a major reason why this disease is being transmitted to children. Increased pertussis incidence among adults is believed to be happening due to the waning of their childhood immunity (7).

A rapid highly specific and sensitive detection of this microbial agent is essential for providing appropriate therapeutic intervention and controlling transmission of the disease. Detection of *B. pertussis* infection by conventional culture provides maximum specificity (8). However, it lacks in sensitivity; the success rate of culture-based detection could be significantly lower than that determined by a serological method especially if samples are from adults or adolescents (7,9). Also, culture of *B. pertussis* is a tedious and time-consuming method. Serological detection (10) has a higher sensitivity than culture, requiring paired samples (acute and

convalescent phase serum), and the convalescent sample is collected at least 3 weeks from the onset of the disease symptoms. Due to this time delay, serological tests are not clinically useful.

Polymerase chain reaction (PCR) provides alternative methods of rapid, highly sensitive, and specific detection for microbial agents including *B. pertussis* (11–15). A presently available instrument, LightCycler™ (Roche Molecular Biochemicals, Mannheim, Germany), is a thermocycler coupled to a fluorescence detection device that allows real-time monitoring of the PCR reaction in progress (16,17). In the LightCycler instrument, samples are subjected to PCR in glass capillary tubes in hot air cycle. The high surface area to volume ratio of the capillary and low thermal mass of air makes rapid temperature transitions possible. Thus, use of this instrument reduces the PCR cycle time duration greatly compared to regular block thermocycler and allows the PCR method to be super rapid. The PCR reaction speed in this instrument is limited only by the DNA polymerase extension time. Since the entire assay can be performed in a closed vessel, use of this instrument

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prevents the possibility of any carry-over contamination as well.

Real-time detection involves the generation of a fluorescence signal during the course of PCR. A rise in signal with increasing cycle indicates amplification. SYBR green I is a fluorescence dye that exhibits increased fluorescence on intercalation in the minor groove of double-stranded DNA. Thus, PCR in the presence of SYBR green I offers a simple method of detection of a target (both qualitative and quantitative) in real time during reaction (18). In monitoring fluorescence while subjecting the PCR end product to increasing temperature, the melting curve of an amplicon DNA can be generated. Melting temperature (T_m) determined from such a melting curve analysis allows the specific detection of a target, since T_m will depend on amplicon's specific sequence composition (particularly GC content) and length. This study reports on *B. pertussis* detection in real time during PCR using SYBR green I in the reaction.

MATERIALS AND METHODS

B. pertussis Control Samples, Clinical Isolates, and DNA Extraction

B. pertussis strain #9797 was obtained from ATCC (American Type Culture Collection, Manassas, VA). The lyophilized cell stock strain supplied in a vial (containing 10^6 CFU equivalent of cell) was first resuspended in 100 μ l of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.3) and then 1:10 serially diluted in the same buffer to prepare control samples. Randomly selected clinical isolates of *B. pertussis* from collections at either the Public Health Laboratory (San Diego, CA) or Iowa State Hygienic Laboratory (Iowa City, IA) were used. DNA from control samples, or clinical isolates of *B. pertussis*, was purified using QIamp Kit (Qiagen Inc., Chatsworth, CA) following a protocol suggested by the manufacturer. Purified DNA was eluted in 100 μ l of elution buffer.

PCR

The sense and antisense primers derived from the repeat sequence region (IS481) of *B. pertussis* genome were identical to those used in previously reported studies (11,15). The PCR mixture contained 2 μ l of extracted DNA or distilled H_2O (for negative control), 0.45 μ M each of sense and antisense primers, 2 μ l of 10X LightCycler-DNA Master SYBR green I (containing Taq DNA polymerase, 10 mM $MgCl_2$, and dNTPs [dATP, dCTP, dGTP, and dUTP instead of dTTP] in the reaction buffer from Roche Molecular Biochemicals, Mannheim, Germany), and 2.4 μ l $MgCl_2$ (25 mM); the

final concentration of $MgCl_2$ in the reaction being 4.0 mM. The final reaction volume was increased up to 20 μ l by adding the required volume of distilled H_2O . The reaction mixture was loaded into the LightCycler™ capillary tubes by centrifugation in a microfuge using appropriate adapters and was subjected to a precycle of 95°C for 30 sec and then amplification was performed using 40 cycles. Each cycle consisted of 94°C for 1 sec, and at 55°C and 72°C for 10 and 15 sec, respectively. During each cycle in the real time of reaction and after completion of primer extension at 72°C, fluorescence (F1) at 520 nm was recorded, while excitation was at 490 nm (programmed internally in the instrument). At the end of 40 cycles of amplification, the reaction was heated to 95°C and then brought down to 35°C. The reaction product was then subjected to a post-PCR melting cycle or was used for analysis by agarose gel electrophoresis. The post-PCR melting cycle consisted of heating up to 98°C at a rate of 0.1°C/sec.

Amplicon Melting and T_m Analysis

Amplicon was subjected to the post-PCR melting cycle, and the melting profile was analyzed from a plot of negative derivative of fluorescence F1 (the emission fluorescence of SYBR at 518 nm) with respect to temperature (T), i.e., $-dF1/dT$ as a function of T. The T_m was determined as the temperature at which the derivative value ($-dF1/dT$) was found to be maximum. Analysis of $-dF1/dT$, as function of T, was performed using software programs provided in the LightCycler instrument itself.

Agarose Gel Analysis

Fifteen percent of the post-PCR product was loaded in an agarose gel (2.5%) containing 0.5 μ g/mL ethidium bromide, and electrophoresis was conducted in TBE (89 mM Tris-borate 2 mM EDTA, pH 8.0) buffer. After the electrophoresis, the DNA bands were visualized through UV transillumination. Amplicon fragment sizes were analyzed with respect to a molecular size marker (100-bp DNA ladder; New England Biolabs, Inc., Beverly, MA).

RESULTS

The amplification reaction was standardized for LightCycler using SYBR green I as the reporter fluorescence signal emitting fluorophore and DNA from control *B. pertussis* strain as the template. Figure 1 shows a real-time fluorescence signal detection plot as a function of cycle number in the reaction using DNA isolated from a serially diluted sample of the *B. pertussis* strain. As expected, fluorescence signal was obtained

Fig. 1. Plot of fluorescence signal (F1) as a function of cycle number in real time during PCR in the presence of SYBR green I using serial (1:10) diluted control *B. pertussis* DNA as template. The dilutions are as indicated on the curves. Negative control had only distilled water in place of DNA.

signifying the presence of amplified DNA at increasing cycle number with increased dilution of template DNA. However, it has to be noted that even the negative control (with no template DNA) showed signal at cycle number 31 and higher. This was due to nonspecific DNA product formation that occurs especially with no, or extremely low, copy number of specific template DNA in the reaction. Thus, following amplification, melting profile analysis was conducted to confirm specific and nonspecific amplification and to determine the T_m of specific product using serially diluted control targets (Fig. 2). At low dilution (10^{-1} to 10^{-4}), amplified product had a single phase melting within a defined melting band (83.5 to 88.5°C) with a peak T_m at 86.5°C. At higher dilutions, melting was biphasic with two melting peaks (T_m s), one around 79–80°C and the other at $86 \pm 0.5^\circ\text{C}$, respectively (Fig. 2a). With control negative, only the melting peak at the lower temperature was observed. Also, the melting peak at the higher temperature ($86 \pm 0.5^\circ\text{C}$) decreased with increased dilution of template DNA. This indicated that under the present reaction condition, T_m for specific DNA product was $86 \pm 0.5^\circ\text{C}$ and for nonspecific product was around 79–80°C. When amplified product was analyzed in agarose gel (inset of Fig. 2a), DNA of expected size (153 bp) was detected, and a decrease in the amount of synthesis was visible at a higher dilution as expected. The result of agarose gel analysis further verified that the higher temperature melting bands were due to the melting of specific DNA product. In agarose

gel, product was detected up to 10^{-6} dilution whereas melting band with peak at 86.5°C was observed up to 10^{-7} dilution, giving a detection limit equivalent to 0.02 and 0.002 CFU, respectively, in the tested volume (2 μl) of sample. Assuming 50% recovery in the process of DNA extraction and a ratio of 100:1 of physical to viable particle in the stock, the analytical sensitivity turns out to be in the range of 1 and 0.1 copies of genomic DNA, respectively, in the tested volume. (Note here that the number of target template DNA sequence per genome is in the range of 100s and extracted DNA is never an intact genome of the bacteria. In addition, a bacteria there could be one full genome and a partially replicated one). This indicated that the sensitivity of detection by melting profile and T_m analysis was about 10-fold better than that detected by agarose gel analysis. This analytical sensitivity of detection by T_m analysis was comparable to that reported in a previous study using a fluorescent stem-loop probe system (15).

The melting profiles ($dF1/dT$ vs. T plot) of amplified DNA of five representative clinical isolates of *B. pertussis* and that of a control negative amplification are analyzed in Fig. 2b. All isolates of *B. pertussis* produced their characteristic single phase melting curve with peak at T_m of $86 \pm 0.5^\circ\text{C}$, whereas the control negative produced a broad single phase melting peak at its T_m of 79.5°C. Thus, isolates were detected and identified from the analysis of the characteristic melting band pattern of PCR amplification targeting IS481 in

Fig. 2. Melting profile analysis by the plot of negative derivative of fluorescence (F1) with respect to temperature (T), i.e., $-dF1/dT$, as a function of T, of the amplified amplicons using serially diluted control *B. pertussis* DNA template (a) or DNA extracted from representative isolates of *B. pertussis* (b). Curves in panel a or lane in inset figure of panel a: no. 2 to 8 represented template DNA dilutions of 10^{-2} to 10^{-7} and no. 9 negative control, respectively. In b, curves no. 1 to 5 represented DNA from five *B. pertussis* isolates. Curve no. 6 was negative control.

the presence of SYBR green I. The amplified DNA fragment of expected size (153 bp) was also distinctly detectable by agarose gel analysis (not shown), and, qualitatively, the DNA fragment band intensities were also in agreement with relative peak heights in the melting bands.

DISCUSSION

This study demonstrated the detection and identification of *B. pertussis* with an additional specificity parameter of the T_m of an amplicon generated by PCR in the presence of SYBR green I in real time during

reaction. A plot of cycle threshold (C_t) number, required to obtain a signal significantly above background as a function of CFU equivalent of the template value at the start of the reaction (Fig. 3) generated from the data of the experiment (Fig. 1), showed a linear relation, suggesting that the assay could be used for the quantitative estimation of the bacteria as well as in a specimen using the intercalator dye SYBR green I in the PCR.

Fluorescent signal arising from a nonspecific product in a PCR-based detection can be reduced to zero using fluorophore labeled hybridization probes, including TaqMan (19) and other fluorescent energy transfer (FET) probes such as a molecular beacon (a stem-loop structured probe) (17,20) or dual linear hybridization probes (21), in the amplification reaction of a microbial agent under investigation. However, appropriate design of all such probes and the optimization of the assay reaction condition is crucial for their application to be successful. In the TaqMan system, the signal is generated by the hydrolysis of the hybridized probe by the advancing Taq DNA polymerase's 5'-3' exonuclease activity. This requires significant holding time at the probe-annealing step for sufficient hydrolysis of the probe to occur, resulting in a compromise in the speed of the reaction and assay. In addition, the TaqMan probe assay does not allow determination of the probe-target melting temperature, which is an additional specificity parameter of detection. Beacon and dual hybridization probes do allow this determination.

PCR-based assays targeting IS481 for *B. pertussis* have been found to have a strong specificity when tested among a large number of relevant gram positive and gram negative bacteria, including the related species excepting *B. holmesii* (11,22). Thus, a PCR method such as the present one targeting IS481 will not exclude amplification of *B. holmesii* as has been observed and reported before (23). A differential detection of *B. pertussis* vs. *B. holmesii* is only possible using a discriminatory stem-loop probe in a PCR-based detection targeting IS481 (17). However, although reported in recent years (24), occurrence of *B. holmesii* infection in pertussis or pertussis-like syndrome is quite rare, and clinical significance of *B. holmesii* infection in the disease process is yet to be unequivocally established. The insertion sequence region IS481 is present in multiple copies (80–100) in *B. pertussis* genome (25), and, thus, can provide the increased sensitivity of an amplification-based assay. In fact, among all the targets tested so far, IS481 is known experimentally to provide the maximum sensitivity of *B. pertussis* detection among other targets such as pertussis toxin (PT) promoter region (26), the porin gene (27), or adenylate cyclase gene (28) of *B. pertussis* genome tested. The present SYBR green I based real-time PCR is technically less involving (no probe design required), less expensive (no cost of probe synthesis), and a super rapid closed vessel assay with minimized chances of contamination. It can be applied for facilitating qualitative or even quantitative detection of *B. pertussis*, the most common and frequently

Fig. 3. Plot of the starting quantity of *B. pertussis* template in terms of log CFU equivalent in a sample on the x-axis and cycle threshold (C_t) on the y-axis. The linearity relation showed feasibility of quantitation of bacteria in a sample. Slope: 3.398. Correlation coefficient: 0.99 as determined by the built-in software program of the instrument.

occurring infection in suspected pertussis cases, and, thus, would be particularly useful for assays conducted in a clinical laboratory setting for a rapid and reliable screening during a *B. pertussis* epidemic. In addition, if required/warranted (i.e., if the purpose is to screen the presence of *B. holmesii* as well), a sample found positive by the present test (thereby eliminating negative samples) can be tested further for discriminatory detection of *B. pertussis* vs. *B. holmesii* using the stem-loop probe based protocol (17).

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