

# Detection and discrimination of *B. pertussis* and *B. holmesii* by real-time PCR targeting IS481 using a beacon probe and probe-target melting analysis

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## Abstract

A beacon probe was designed to detect one of the two documented single nucleotide changes in IS481 target allele of *Bordetella holmesii* genome as compared to *Bordetella pertussis*. PCR amplified product targeting a region of IS481 in presence of the probe was subjected to a post-PCR hybridization and melting cycle. Hybrid of the probe with *B. pertussis* specific target had a different thermal stability than that with allele having the single nucleotide change in *B. holmesii*. The melting of *B. pertussis*-probe hybrid occurred in a single phase; while that of *B. holmesii*-probe hybrid was biphasic-one for allele identical to that in *B. pertussis* and the other for that with a single nucleotide change in *B. holmesii* genome, with a difference in melting temperature ( $T_m$ ) of 6.5 °C. The characteristic melting profile and  $T_m$  analysis was the basis for discriminatory detection of *B. pertussis* from *B. holmesii*. The method was applied in a representative set of clinical isolates of *B. pertussis* and *B. holmesii* and the result was in agreement with conventional culture method.

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**Keywords:** *Bordetella pertussis*; *Bordetella holmesii*; IS481; PCR; Melting temperature

## 1. Introduction

Pertussis (whooping cough) is caused primarily by *Bordetella pertussis* infection. It continues to be a significant respiratory disease among unvaccinated children [1] and young adults whose immunity acquired through childhood vaccination or infection has waned [2,3]. *Bordetella holmesii* a closely related species of *B. pertussis* was found previously associated with septicemia in immunocompromised patients [4]. It has been isolated during recent years from patients with pertussis-like symptoms [5–7]. However, the clinical significance of *B. holmesii* as a respiratory agent and whether or not it does cause the disease is yet to be established unequivocally; albeit so far there is at least one recent report on *B. holmesii* infection in a previously healthy immunocompetent patient, causing respiratory illness [8]. A rapid discriminatory detection of *B. pertussis* and *B. holmesii* with high specificity and sensitivity would permit accurate diagnosis

and such differential diagnostics will be of further importance in the understanding of epidemiology and clinical significance of *B. holmesii* in pertussis-like illness.

Detection and identification of organisms like *B. pertussis* or *B. holmesii* by conventional culture although very specific is labor intensive, difficult to grow, and time consuming (needs several days sometimes weeks to grow). Also culture assay often is not sensitive enough and results in a high proportion as false negative especially if the samples are from young adults or adolescents [9,10]. Serological tests although may have higher sensitivity, is not clinically useful due to necessary time delay (to acquire convalescent sera). Also serological tests are not all standardized, nor routinely provided by most clinical laboratories.

In view of its enormous sensitivity and specificity rapid PCR based detection of *B. pertussis* has attracted much attention in recent years. The chromosomal regions that have been used as targets for *B. pertussis* specific PCR include (i) the adenylate cyclase toxin (ACT) gene [11], (ii) a region upstream of the porin gene [12], (iii) pertussis toxin (PT) promoter region [13], and (iv) repeat insertion

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sequences [14–16]. Among these, repeat insertion sequence IS481 region being present in multiple copies (80–100) in *B. pertussis* [17], is a target of choice for amplification and detection with greater sensitivity. It is documented that genomic sequence of *B. holmesii* also contains regions homologous to IS481 [18]. Thus PCR targeting IS481 is expected to generate positive DNA product for both *B. pertussis* and *B. holmesii* as observed before [19]. As a result it although will provide high sensitivity of detection, will lack the ability to detect differentially one species versus the other. However, since there are two single nucleotide changes (A/C and C/T variation) in alleles of *B. holmesii* genome; one approach to take advantage of this target (IS481) for enhanced sensitivity and performing discriminatory detection of *B. pertussis* versus *B. holmesii* is to exploit the single nucleotide differences in alleles of IS481 in the genome of *B. holmesii* as compared to that in *B. pertussis*.

DNA probes are particularly useful for detection of a PCR amplified target sequence with high specificity and sensitivity [20–22]. Molecular beacon is a stem-loop structured DNA probe; its principle of action has been described in details in previously published reports [23,24]. In brief, it possess a fluorescent dye and a quencher molecule at 5' and 3' ends, respectively, and is designed to form stem-loop structure that brings into close proximity the fluorescent dye and quencher of the probe, resulting in minimal fluorescence. In presence of a complementary target sequence, the probe will hybridize to the target, separating the reporter dye from the quencher, resulting in a measurable increase in fluorescence. Importantly, the non-hybridized probe fraction regains its original conformation fast, when the solution containing the probe and target DNA is brought to an appropriate low temperature (lower than the melting temperature of stem structure) from a hybridization temperature, and will not contribute any fluorescence. In a PCR containing such a probe, probe-target hybrid will form as the temperature suitable for hybrid formation is reached in the temperature cycling and fluorescence will be emitted only from the fraction of the probe involved in hybridization. Thus a PCR in presence of a beacon probe and subsequent fluorescence analysis will allow the detection of a target immediately after the target is amplified, requiring no additional steps. Also, since the strength of a probe-target hybridization depends on the degree of probe-target sequence matching, analysis of melting profile of a probe-target hybrid and its melting temperature ( $T_m$ ) can allow differentiating between targets with sequence differences even by a single nucleotide [23].

This communication reports detection discriminating *B. pertussis* from *B. holmesii* using PCR amplification of IS481 region as a target, and a beacon probe. The differences in thermal stability of probe-target hybrid determined from melting profile and  $T_m$  analysis was the basis for differentiating *B. pertussis* from *B. holmesii*.

## 2. Materials and methods

### 2.1. *B. pertussis* and *B. holmesii* control samples, clinical isolates and DNA extraction

*B. pertussis* strain #9797 and *B. holmesii* strain #51541 were obtained from ATCC (American Type Culture Collection, Manassas, VA, USA). The strains were supplied in vials containing  $10^6$ – $10^7$  CFU equivalent of cell lyophilized (CFU value was provided by ATCC). The entire lyophilized cell stock of each strain was first re-suspended in 100  $\mu$ l of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.3) and then 1:10 serial diluted in the same buffer to prepare control samples. Clinical isolates of *B. pertussis* used were provided either by Public Health Laboratory at San Diego, CA, USA or Iowa State Hygienic Laboratory, Iowa City, Iowa, USA. *B. pertussis* isolates were sub-cultured on Regan-Lowe agar plates with 40  $\mu$ g/ml of cephalexin and when colonies grew, a single colony of each isolate was suspended in 100  $\mu$ l of TE buffer. A crude DNA extract of each isolate of *B. holmesii* used in this study was received from Dr. Loeffelholz of Iowa State Hygienic Laboratory (Originally *B. holmesii* isolates were provided by Dr. H. George, of Massachusetts Department of Public Health to Dr Loeffelholz). DNA from a suspension of control or clinical isolate of *B. pertussis* or *B. holmesii* (crude DNA extract) was purified using QIamp kit (Quiagen Inc, Chatsworth, CA, USA) following a protocol suggested by the manufacturer. Purified DNA was eluted in 100  $\mu$ l of elution buffer.

### 2.2. Primers for PCR and beacon probe design

The sense primer (5'GAT TCA ATA GGT TGT ATG CAT GGT T3') identical to that used in previous studies [14,25], corresponding to nucleotide #22–46 (GenBank accession no. M28220; *B. pertussis* sequence) or nucleotide #1–25 (GenBank accession no. AF349431; *B. holmesii* sequence) and a modified antisense primer (5'GGC ACA CAA ACT TGA TGG GCG A 3') corresponding to nucleotide #198–177 (GenBank accession no. M28220) or nucleotide #177–156 (GenBank accession no. AF349431) of IS481 sequence region, respectively, were used. The molecular beacon probe (5'-FAM **CG GACCTTCTACGTCGC**\*CTCGAAATG **GTCC** G BHQ-3') was synthesized and purified by Integrated DNA Technologies, Inc (Coralville, IA, USA). The underlined sequence except the nucleotide marked with star (should be 'A' for perfect match), was complementary to *B. pertussis* genome (nucleotide #139–167; GenBank accession no. M28220) and to certain alleles of *B. holmesii* (nucleotide #118–146; Gen Bank accession no. AF349431). But it was in exact match with other alleles in the genome of *B. holmesii* (GenBank accession no. AF349431). In the absence of any target the stretch of six nucleotides (in bold) from 5' and 3' ends formed the stem

structure. FAM (Fluorescein) and BHQ (Black Hole Quencher) were used as the fluorophore and quencher, respectively.

### 2.3. PCR

The 20  $\mu$ l PCR mixture contained 2  $\mu$ l of extracted DNA or distilled H<sub>2</sub>O (for negative control), 0.3  $\mu$ M each of sense and antisense primers, 0.2 mM each of dNTPs (dATP, dCTP, dGTP, dTTP), 3.0 mM MgCl<sub>2</sub>, 0.5 U of Taq DNA polymerase in 1  $\times$  PCR buffer (Perkin Elmer Inc) and 50 nM of beacon probe. The reaction mixture was added in the capillary tube of LightCycler instrument (a thermocycler coupled with a fluorescence detection device; from Roche Molecular Biochemicals, Mannheim, Germany) and was subjected to a pre-cycle of 95 °C for 30 s and then amplification was performed using 45 cycles. Each cycle consisted of 94 °C for 1 s, 58 and 72 °C each for 15 s. At the end of amplification the reaction was heated to 95 °C and brought to 25 °C at a cooling rate of 0.1 °C/s. In this post amplification cycle amplicons and probe completely denatured and the target strand of an amplicon hybridized to the probe as the temperature gradually dropped to 25 °C.

### 2.4. Probe-target melting and melting temperature ( $T_m$ ) analysis

Once it reached 25 °C the probe-target melting profile was studied by subjecting the reaction to gradual heat up to 95 °C at a rate of 0.1 °C/s. The melting profile was analyzed from a plot of fluorescence  $F1$  (at 518 nm the emission maximum for fluorescein) signal or  $-dF1/dT$  (negative derivative of fluorescence ( $F1$ ) with respect to temperature ( $T$ )) as a function of  $T$ . The melting temperature ( $T_m$ ) was determined as the temperature at which the derivative value ( $-dF1/dT$ ) was maximum. Fluorescein attached to a probe was excited at 490 nm (programmed internally in the instrument). All analysis of  $F1$  or ( $-dF1/dT$ ) as function of  $T$  was performed using software programs provided in the LightCycler instrument itself.

### 2.5. Agarose gel analysis

The post-PCR product was loaded in an agarose gel (2.5%) containing 0.5  $\mu$ 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 trans-illumination. Amplicon sizes were analyzed with respect to molecular size marker (100 bp DNA ladder, from New England Biolabs Inc, Beverly, MA, USA).

## 3. Results

### 3.1. Melting profile and $T_m$ analysis of control *B. pertussis* and *B. holmesii* amplified product

The plot of ( $F1$ ) or ( $-dF1/dT$ ) as a function of temperature ( $T$ ), as amplification product using template DNA sample of control *B. pertussis* and control *B. holmesii* strains was subjected to gradual increase in temperature after completion of post-PCR hybridization cycle, is shown in Fig. 1. At low temperature (30 °C), fluorescence value ( $F1$ ) of both *B. pertussis* and *B. holmesii* amplification was 4–5 fold higher compared to that of negative control amplification (no template DNA, probe alone) indicating the presence of the targets in the form of a target-probe hybrid. As the temperature increased over 60 °C until 80 °C, negative control showed increased fluorescence due to the melting of stem structure of the probe. *B. pertussis* specific target-probe showed a sharp decrease in fluorescence from 65 to 70 °C, whereas fluorescence of *B. holmesii* specific target-probe decreased in two phases (65–70 and 73–77 °C). These changes in fluorescence were due to the melting of the probe from the targets, and ( $-dF1/dT$ ) vs  $T$  plot correspondingly generated a single peak at 68 °C ( $T_m$ ) and two peaks (68 and 74.5 °C;  $T_m$ ) for *B. pertussis* and *B. holmesii*, respectively. At higher temperatures the fluorescence profile for both *B. pertussis* and *B. holmesii* merely reflected that of probe alone. To be noted that  $F1$  signal goes up due to melting of stem structure of the free probe whereas it goes down due to melting of the probe from a target. Thus ( $-dF1/dT$ ) vs  $T$  curve for negative control generated a reverse melting band (within a broad temperature range 60–80 °C) with a peak at 72 °C (its  $T_m$ ). The beacon probe sequence had one nucleotide mismatch with *B. pertussis* but was perfectly complementary to some alleles of IS481 target in the genome of *B. holmesii*. Thus hybrid formed by the probe with *B. pertussis* target or alleles that were identical to *B. pertussis* in *B. holmesii* genome had same thermal stability a  $T_m$  of 68 °C. Whereas other alleles in *B. holmesii* being perfect complementary to probe, formed probe-target hybrid of higher thermal stability and thus had a higher  $T_m$  (74.5 °C).

The melting profiles of PCR product using DNA from serial diluted control strains of *B. pertussis* and *B. holmesii* are shown in Figs. 2 and 3, respectively. In  $F1$  vs  $T$  plot, at low temperature (30 °C)  $F1$  value decreased with increasing dilution of both *B. pertussis* and *B. holmesii* and  $F1$  value was higher than that of negative control for up to 10<sup>-8</sup> dilution (Figs. 2a and 3a). This indicated that respective targets were detectable by probe-target hybrid formation up to 10<sup>-8</sup> dilution. The characteristic decrease of  $F1$  in single phase (65–70 °C) for *B. pertussis* or biphasic (65–70 and 73–77 °C) for *B. holmesii* at higher dilutions (higher than 10<sup>-6</sup> for *B. pertussis* and higher than 10<sup>-4</sup> for *B. holmesii*) were not, however, very apparent in  $F1$  vs  $T$  profile. This was due to melting (within a broad

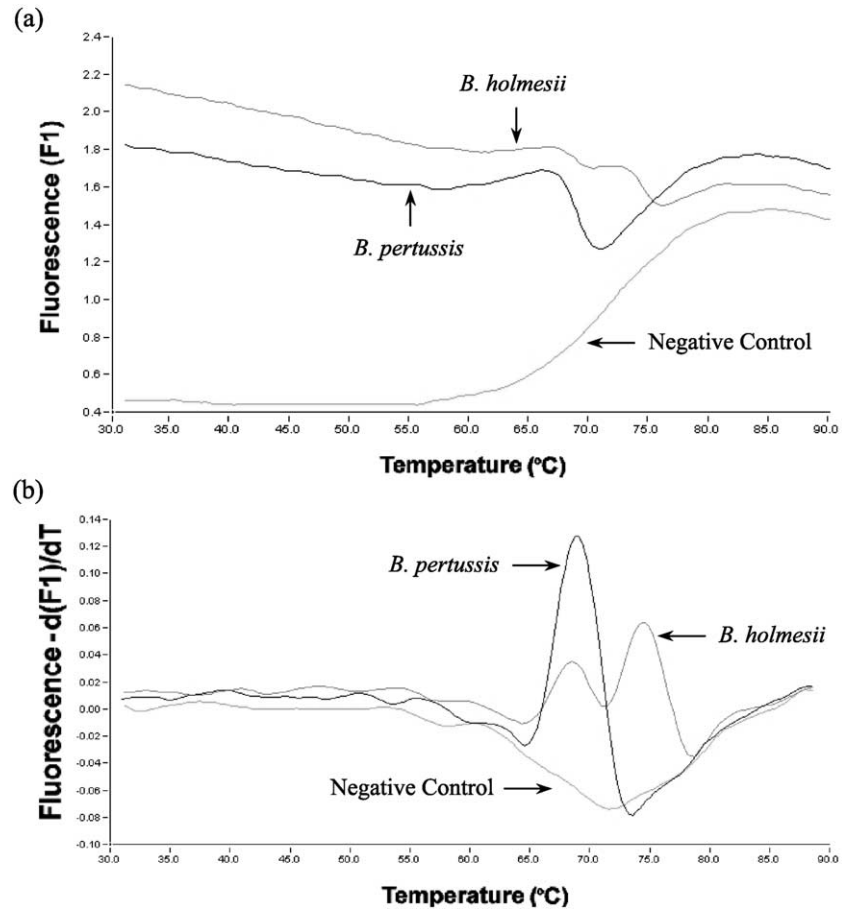


Fig. 1. The melting profile of probe-target hybrid by the plot of fluorescence ( $F1$ ) vs. temperature ( $T$ ) (a) or by  $(-dF1/dT)$  vs.  $T$  (b), using PCR product of a control isolate of *B. pertussis* and *B. holmesii*.

temperature range 60–80 °C) of stem structure of free probe in increased amount that contributed to  $F1$  in opposite direction of that due to probe-target melting. In  $dF1/dT$  vs  $T$  plot the peak height of the curves at 68 °C for *B. pertussis* and that at 68 °C and 74.5 °C for *B. holmesii* decreased with increased dilution systematically. For *B. pertussis* a well defined melting peak was observed up to  $10^{-7}$  dilution and there was a shoulder for  $10^{-8}$  dilution at 68 °C the characteristic  $T_m$ . Whereas for *B. holmesii* the two peaks at 68 and 74.5 °C were defined up to  $10^{-6}$  dilution; for  $10^{-7}$  and  $10^{-8}$  dilution peak at 74.5 °C was detected but not that at 68 °C. The amplification of negative controls showed only a reverse peak at its  $T_m$  of 72.5 °C as expected. This indicated that by fluorescence melting profile (in a  $dF1/dT$  vs  $T$  plot) and  $T_m$  analysis the sensitivity of detection was at least up to  $10^{-7}$  and  $10^{-6}$  dilution of stock, respectively, for control *B. pertussis* and *B. holmesii* strains used. The insets in Figs. 2 and 3 show the agarose gel analysis of the amplified DNA products for the serially diluted cells. DNA fragment of expected size (177 bp) for both *B. pertussis* and *B. holmesii* was amplified and the band intensities decreased gradually with increased dilution and were detectable up to  $10^{-7}$  dilution. This gel analysis data verified that the observed

fluorescence melting-pattern was due to the presence of respective target DNAs, the amount of which decreased with increased dilution as expected. Also the sensitivity of detection of *B. pertussis* and *B. holmesii* by probe-target melting profile and  $T_m$  analysis was about equivalent to that by agarose gel analysis.

### 3.2. Detection of clinical isolates by melting profile and $T_m$ analysis

Melting profiles ( $dF1/dT$  vs  $T$  plot) of amplified DNA (in presence of the probe) of clinical isolates of *B. pertussis* and *B. holmesii* six of each and that of a control negative and a positive amplification were analyzed in Fig. 4. All isolates of *B. pertussis* produced its characteristic single phase melting curve with peak at  $T_m$  of 68 °C (Fig. 4a) and all *B. holmesii* a biphasic melting with  $T_m$  at 68 °C and at  $74 \pm 0.5$  °C, respectively (Fig. 4b), whereas control negative produced a broad single phase melting with a reverse peak at its  $T_m$  of 72.5 °C (Fig. 4a and b). Thus isolates were detected and identified discriminating one strain (*B. pertussis*) from the other (*B. holmesii*) from analysis of characteristic melting band pattern of PCR amplification targeting IS481 in presence of the beacon

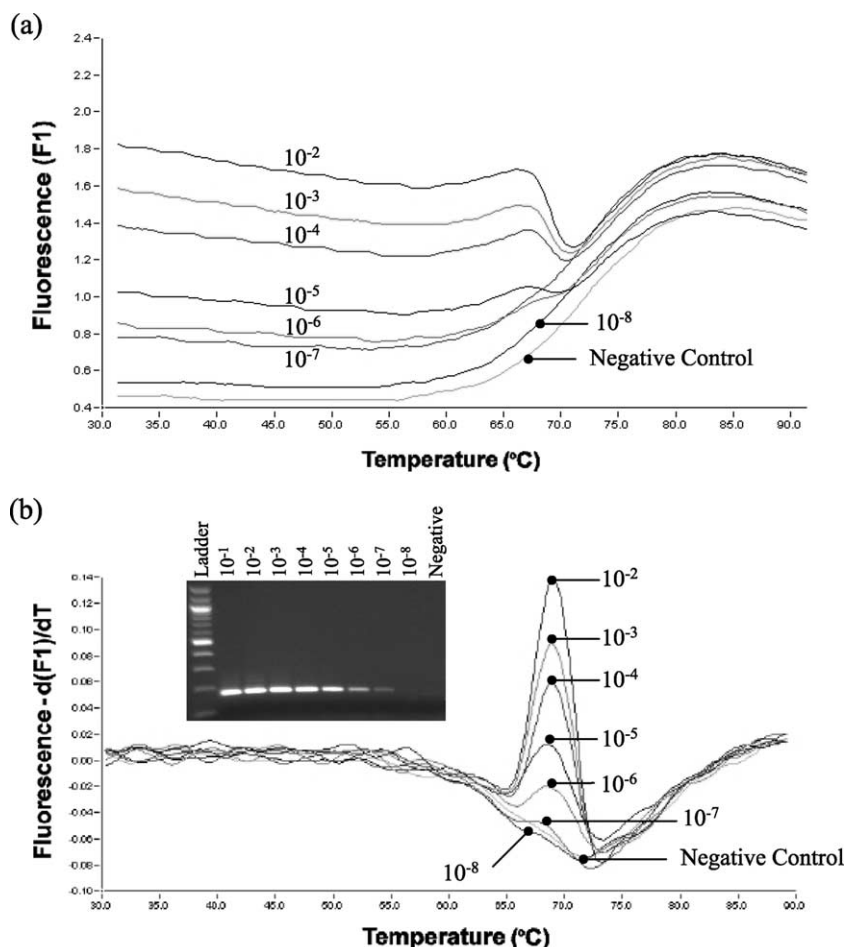


Fig. 2. The melting profile of probe-target hybrid by  $F1$  vs  $T$  (a) or by  $(-dF1/dT)$  vs  $T$  (b) using PCR product of serial (1:10) diluted control isolate of *B. pertussis*. Inset: analysis of post-PCR product by agarose gel electrophoresis. Ladder: 100 bp molecular size markers. The dilutions of control samples were as indicated on the melting curves or on top of the agarose gel lanes.

probe. The amplified DNA fragment (177 bp) was also distinctly detectable by agarose gel analysis (inset in Fig. 4). Qualitatively the DNA fragment band intensities were also in agreement with relative peak heights in the melting bands. However, targeting *IS481*, amplified DNA fragment sizes being the same for both *B. pertussis* and *B. holmesii* discriminatory detection was not possible by agarose gel analysis as expected and observed previously [19].

#### 4. Discussion and conclusion

The quality and performance efficiency of a beacon probe depends on signal to noise ratio and on its efficiency of quenching. The molecular beacon probe used in this study was characterized (not shown) with respect to its signal to noise ratio and efficiency of quenching following the method described in previously published report [22] and were found to be 28:1 and  $\sim 97\%$ , respectively. These values were better than those of other beacon probes used in studies reported before [14,22,24]. This improved quality parameter of the probe may be partly due to use of BHQ

instead of DABCYL as the quencher and partly due to the sequence organization of stem and probe part of the beacon probe (four nucleotides at 5' and five nucleotides at 3' that formed stem were common to probe part).

A beacon probe due to the intra-molecular competition between internally base paired stem and pairing of the loop with an external target is known to provide higher specificity compared to a linear hybridization probe. The stability of a beacon probe-target hybrid depends on the length of hybridizing sequence, stem sequence, GC content, and location of any mismatch in the sequence. By appropriate designing of the stem for a hybridizing sequence in a beacon, the difference in  $T_m$  due to a particular mismatch can be made higher in a beacon probe-target compared to a linear probe-target. In fact, using LightCycler linear hybridization probe targeting the same mismatch (A/C variation, the one detected in the present report) in *B. pertussis* vs *B. holmesii* only a change of 1–1.5 °C for  $T_m$  value was detected (personal communication from Dr Udo Reischl, University of Regensburg, Regensburg, Germany). The beacon probe used in the present study introduced a difference in  $T_m$  of 6.5 °C. This difference was sufficient

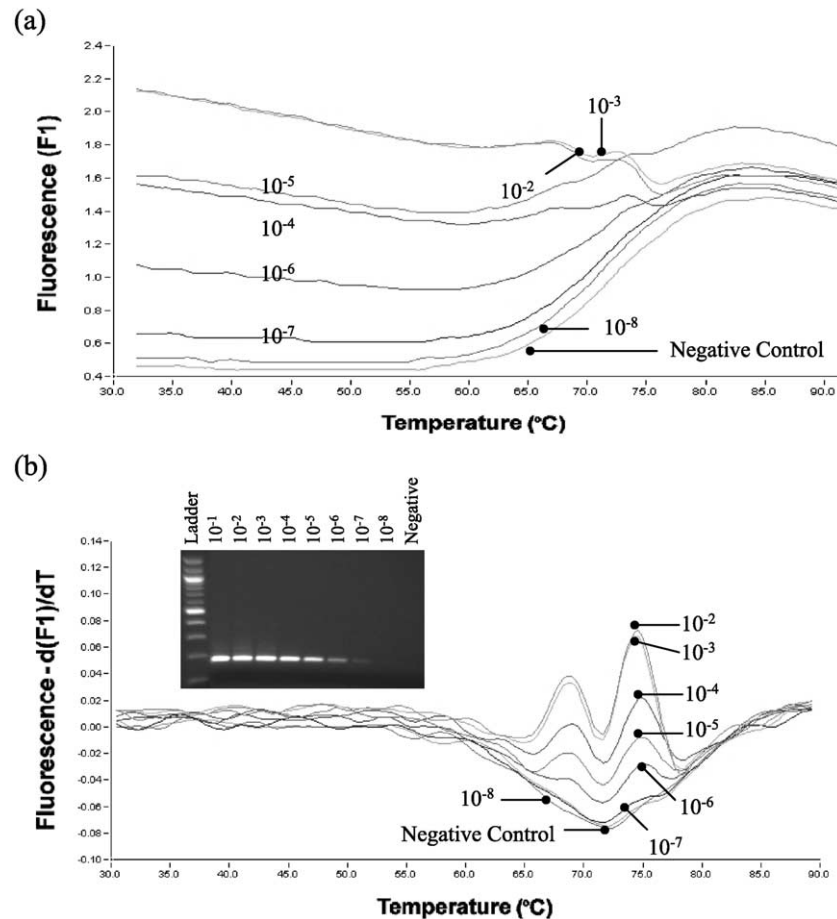


Fig. 3. The melting profile of probe-target hybrid by  $F1$  vs.  $T$  (a) or by  $(-dF1/dT)$  vs.  $T$  (b) using PCR product of serial (1:10) diluted control isolate of *B. holmesii*. Inset: analysis of post-PCR product by agarose gel electrophoresis. Ladder: 100 bp molecular size markers. The dilutions of control samples were as indicated on the melting curves or on top of the agarose gel lanes.

enough to produce a distinct biphasic-melting pattern as well. Also, on analysis of the melting bands for *B. holmesii* isolates used in this report, it was determined that the average ratio of peak heights (data not shown) at 68 °C to that at 74.5 °C was in the range of 1:2–1:2.6. This data indicated that two alleles (allele identical to that in *B. pertussis* and the allele with nucleotide A/C change) are present in *B. holmesii* genome in above ratio range (width being approximately same for both melting bands).

Since plot of  $(-dF1/dT)$  vs  $T$  had better resolution than  $F1$  vs  $T$ , melting profile of all clinical isolates were analyzed using  $(-dF1/dT)$  vs  $T$  curves only. The tested set of clinical isolates of *B. pertussis* and *B. holmesii* were representatives of collection from patients with pertussis from two cities (San Diego, CA and Iowa City, Iowa) and patients with pertussis like symptoms in the state of Massachusetts, MA, respectively, during last few years. The result of the present method of discriminatory detection in these isolates was in agreement with conventional culture. This indicated that the beacon probe and the experimental condition used potentially should be applicable for successful detection and identification of a broader range of *B. pertussis* and *B. holmesii* samples.

Insertion sequences of eubacteria are often transposable elements, capable of spontaneous elimination and undergoing horizontal transfer across species [27,28]. Thus theoretically absolute specificity targeting IS481 like sequences can never be expected considering such evolutionary perspective of genome organization. However, from practical consideration, PCR based assays targeting IS 481 developed for *B. pertussis* so far has been found to have strong specificity when tested among large number of relevant gram positive and gram negative bacteria including the related species excepting *B. holmesii* [18,25]. Sensitivities of assay targeting IS481 have also been found to be the highest of all the PCR targets used [10]. Thus successful use of PCR based assays targeting IS481 continues to be reported during recent years [14,18,19,26]. However, it will be prudent to evaluate results of PCR from IS481 region by comparing that of another target in the genome. This will help to rule out the possibility of any false interpretation of an assay particularly with an unknown clinical sample.

Sensitivity of the present beacon probe based discriminatory assay could be delineated performing melting profile analysis in serial diluted control strains (Figs. 2b and 3b). Considering titer ( $10^4$ – $10^5$  CFU/ $\mu$ l) of stock strains,

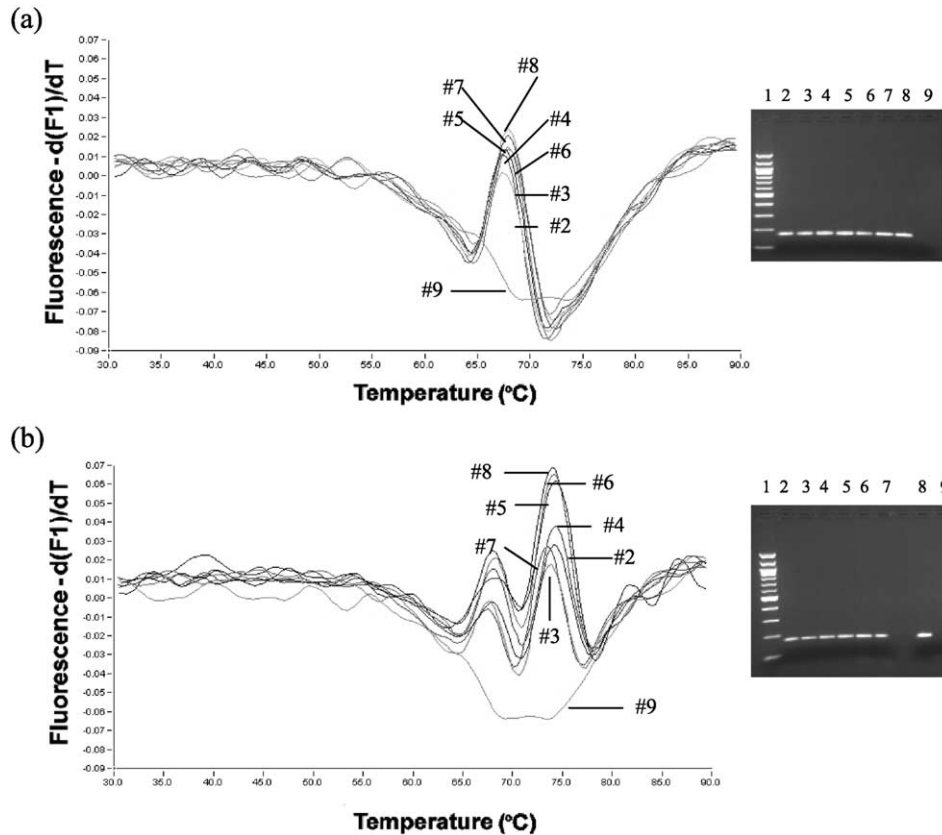


Fig. 4. The melting profile by the plot of  $(-dF1/dT)$  vs.  $T$  for probe-target hybrid using PCR product of clinical isolates of *B. pertussis* (a) and *B. holmesii* (b) as targets. Inset: analysis of post-PCR product by agarose gel electrophoresis. Isolates are #2–7; #8: positive control; #9: negative control.

sensitivity of detection was 0.02–0.2 CFU for *B. holmesii* (corresponding to  $10^{-6}$  dilution) and 0.002–0.02 CFU for *B. pertussis* (corresponding to  $10^{-7}$  dilution) in the tested volume ( $2 \mu\text{l}$ ) of sample. This sensitivity range was comparable to that published in a previous report [11].

In conclusion, it has been demonstrated using a limited number of isolates that presence of a single nucleotide change in IS481 can be detected using an appropriately designed molecular probe and post PCR probe-target melting profile analysis, allowing a discriminatory detection of *B. pertussis* vs *B. holmesii*. Using the present method analysis of additional geographically and temporally diverse isolates is important. This is to date the only molecular assay targeting IS 481 to be reported that is capable of distinguishing *B. pertussis* and *B. holmesii* with a significant resolution.

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