Molecular and Cellular Probes (2001) **15**, 161–167
doi:10.1006/mcpr.2001.0357, available online at http://www.idealibrary.com on IDEAL®

Bordetella pertussis detection by spectrofluorometry using polymerase chain reaction (PCR) and a molecular beacon probe

S. K. Poddar* and C. T. Le

DoD Center for Deployment Health Research, Naval Health Research Center, San Diego, CA 92186-5122, USA

(Received 15 December 2000, Accepted 6 March 2001)

Bordetella pertussis was detected by spectrofluorometry following PCR incorporating a molecular beacon probe in the reaction. A DNA fragment from the tandem repeat sequence region (IS*481*) of the genome of *B. pertussis* was amplified in presence of the probe complementary to an internal segment of the amplified DNA fragment. Fluorescein (FAM) and DABCYL were used as the fluorophore and quencher in the probe. The probe was characterized for its signal to noise ratio by homogeneous solution hybridization with a complementary oligonucleotide. Measurement of fluorescent signal at the emission maxima of FAM, immediately after a PCR was used to detect the *B. pertussis* target, with no additional steps. Presence of *B. pertussis* in a sample was also examined by agarose gel electrophoresis of the PCR product. A serial diluted stock of *B. pertussis* (ATCC strain #9797) and fourteen clinical isolates of *B. pertussis* were examined. The sensitivity of detection by fluorescent measurement was found to be at least in the range of 0·01–0·1 CFU per 10 μl of the sample and was equal to or better than that detected by agarose gel analysis.
© 2001 Academic Press

KEYWORDS: *B. pertussis*, fluorescein, DABCYL, quencher, spectrofluorometry, PCR.

INTRODUCTION

Bordetella pertussis is the causative agent of pertussis, a well-known childhood respiratory disease. Immunization with pertussis vaccines has controlled the disease significantly in children. However, studies in civilian and military populations suggest that *B. pertussis* is emerging as a frequently occurring infection among adolescents and young adults,[1–3] most likely due to waning of their childhood immunity. Rapid and highly reliable (highly specific and sensitive) laboratory diagnosis of the disease is important for providing early appropriate therapeutic intervention, and controlling the disease transmission. Diagnosis by culture is insensitive, and depends upon the viability of *B. pertussis*, which in turn depends on the time of specimen collection relative to the course of infection, specimen transportation, and storage conditions, among other factors.[4,5] Diagnosis by serology has a higher sensitivity than culture; however serological tests may not be clinically useful as convalescent specimens are preferred, are not standardized, and not routinely provided by clinical laboratories.

The polymerase chain reaction (PCR) provides an alternative method for rapid detection of a microbial agent in question.[6–10] However, in a prototypical PCR, the detection of the amplified DNA target with higher

* Author to whom all correspondence should be addressed at: DoD Center for Deployment Health Research Center, Naval Health Research Center, Building 335, P. O. Box 85122, San Diego, CA 92186-5122, USA. Tel: +1 619 553 0755; E-mail: poddar@nhrc.navy.mil

0890–8508/01/030161+07 $35.00
© 2001 Academic Press

specificity and/or sensitivity requires a multistep procedure involving hybridization with an appropriately labelled probe.[7] Molecular beacon[10–12] is a DNA-probe system, consisting of a stem-loop structure conjugated with a fluorophore at one end of the stem and a quencher on the other, with the intervening loop composing the probe sequence. The molecular principle of its action has been explained in previous reports.[11,12] In brief, a beacon probe in its original conformation has the fluorophore and the quencher very close to each other. In response to an excitation light, the fluorescence emitted from the fluorophore gets readily adsorbed by the quencher because of their close proximity and thus effectively does not emit fluorescence. When the probe finds its specific target, its conformation is changed from stem-loop to linear target-probe hybrid molecule (the target-probe structure is energetically favored over the original stem-loop structure of the probe). In the probe–target hybrid conformation, the fluorophore and the quencher moves apart a distance equal to the stretch of nucleotide sequence between them, allowing unperturbed emission of fluorescence from the fluorophore in response to an excitation light. Importantly, the probe fraction that does not hybridize regains its original conformation fast, when the solution containing probe and target DNA is brought from a high hybridization temperature to an appropriate low temperature (lower than the melting temperature of stem structure). The unhybridized probe then does not contribute any fluorescence. The detection of a target PCR product is therefore possible by spectrofluorometry immediately after it is amplified, in presence of such a probe in the reaction, requiring no additional steps.

The present report is a study on the detection of *B. pertussis* using PCR in presence of a molecular beacon and subsequent fluorescence analysis. The sensitivity of detection by fluorescence analysis was compared with that obtained by agarose gel analysis. This beacon probe based PCR-fluorometric method was tested on clinical isolates.

MATERIALS AND METHODS

Bordetella pertussis control samples, clinical isolates and nucleic acid (DNA) extraction

Bordetella pertussis strain #9797 was obtained from ATCC (American Type Culture Collection, Manassas, VA, USA). It was supplied in a vial containing 10^6–10^7 CFU equivalent of cells lyophilized (CFU value was provided by ATCC). The entire lyophilized cell stock in the vial was first resuspended in 100 µl of TE buffer (10 mM Tris, 1 mM EDTA (pH 8·3)) and then serially diluted (1:10) in the same buffer. The serial diluted cells were used as the control samples. Clinical isolates of *B. pertussis* were provided by the San Diego County, Public Health Laboratory at San Diego, CA, USA. Each isolate was sub-cultured on Regan-Lowe agar plates. When colonies were visible, bacteria from a single colony for each isolate was suspended in 100 µl of TE buffer. DNA was extracted from each control or clinical sample suspension (100 µl) using QIamp kit (Quiagen Inc, Chatswort, CA, USA) following the manufacturer's suggested protocol. The DNA for each sample was collected in 100 µl of the elution buffer.

Probe design and preparation

The specific molecular beacon probe (5′ FAM CCTAGA GATGAACACCCATAAGC ATGC CCG TCTAGG DABCYL 3′) was designed with a 24-nt loop sequence (not underlined) complementary to an internal *B. pertussis* IS*481* sequence. The six nucleotide (underlined) sequences formed the stem structure. 5′–6 fluorescein (FAM) and DABCYL were used as the fluorophore and quencher. A commercial company (Synthetic Genetics, San Diego, CA, USA) synthesized and purified the beacon probe.

PCR

The sense and antisense primers derived from the tandem repeat sequence region (IS*481*) of the *B. pertussis* genome, were identical to those used in a previous study[7]. The 100 µl mixture for PCR with or without 0·125 µM of beacon probe contained, 10 µl of extracted nucleic acid (DNA), 0·2 mM each of four dNTPs (dATP, dCTP, dGTP, dTTP), 0·3 µM of each primer, 3·0 mM $MgCl_2$, and 2·5 U of *Taq* DNA polymerase in 1X PCR buffer (Perkin Elmer, Inc.). The reaction was subjected to one pre-cycle of 95°C for 2 min. Amplification was performed using 40 cycles. Each amplification cycle was: 94°C for 10 s, 58°C for 30 s, and 72°C for 30 s. Then the reaction went through a post-cycle consisting of 95°C for 1 min, 70°C for 2 min, 60°C for 2 min, 55°C for 2 min, 50°C for 2 min, and was then cooled down to room temperature.

Fluorescence analysis

The fluorescence emission either at a fixed wavelength (518 nm, the emission maxima for fluorescein)

or a scanned spectrum (500–600 nm) was obtained with an excitation at 490 nm using a model LS 50B spectrofluorometer (Perkin Elmer). Samples were analysed either in a micro-cuvette or in multiple well plate readers. The fluorescence of a sample (probe plus possible amplified DNA) found greater than or equal to ($F_n + 3SD$) was considered significant above the background, where F_n was the average fluorescence of three negative control (having the same amount of probe only as in the sample) and SD, their standard deviation.

Molecular beacon probe characterization

A solution mixture containing 50 nM probe and 500 nM of complementary oligonucleotide target was prepared and incubated at room temperature for 1 h to allow hybridization. The mixed sample was then analysed for fluorescence by scanning emission spectrum. The efficiency of quenching (E_{ff}) of the beacon probe was determined by fluorescence analysis using the following formula: $E_{ff} = \{1 - (F_{uh} - F_b)/(F_{ch} - F_b)\} \times 100$; where F_{uh} and F_{ch} were the fluorescence intensity of unhybridized and completely hybridized probe respectively and F_b was the background fluorescence of buffer measured at 518 nm the emission maximum of FAM. The signal to noise ratio was determined by $(F_{ch} - F_b):(F_{uh} - F_b)$.

Agarose gel analysis

Ten percent of the PCR product was analysed by electrophoresis in TBE buffer (89 mM Tris-borate, 2 mM EDTA (pH 8·3)) in 2% (w/v) agarose gel containing 0·5 µg/ml ethidium bromide. The DNA bands were visualized through an U.V. transilluminator. Molecular size marker (100-bp DNA ladder) used was obtained from New England Biolabs, Inc; Beberly, MA, USA.

RESULTS

Molecular beacon probe characterization

Fig. 1 shows the characteristic emission spectrum of the probe before hybridization (probe alone, curve A) and after hybridization (mixed sample, curve B) with the complementary target oligonucleotide. The curve A was reproduced when the probe was mixed with an oligonucleotide with a sequence unrelated to the target. The fluorescence intensity measured at 518 nm (emission maxima), increased about 18–20-fold on hybridization with complementary target. The fluorescence intensity did not increase further on addition of excess target oligonucleotide >500 nM. The efficiency of quenching (E_{ff}) of light emitted from fluorophore by the quencher in this probe preparation was >95%.

Amplification of control samples of *B. pertussis* and detection

Fig. 2 shows the spectral analysis of *B. pertussis* control samples (10^{-4} to 10^{-9} dilutions) following PCR in presence of the probe. The intensity of fluorescence at 518 nm decreased with increasing dilution of *B. pertussis* cell to 10^{-8}. The fluorescence intensities of 10^{-8} and 10^{-9} diluted samples were similar, and were significantly greater than that from control negative sample. The inset in Fig. 2 shows the agarose gel analysis of the amplified DNA products for the serially diluted cells. DNA fragments of expected size (153 bp) were amplified and the DNA band intensities were detectable up to the 10^{-7} dilution, but decreased gradually from the 10^{-4} dilution. The gel electrophoresis data further verified that the observed fluorescence of the samples at 518 nm were due to the presence of amplified DNA, the amount of which decreased with increased dilution of the sample as expected. Considering the initial concentration (10^4–10^5 CFU/µl) of the control *B. pertussis* stock, the sensitivity of detection by agarose gel analysis was determined to be in the range of 0·01 to 0·1 CFU in the tested volume. The sensitivity of fluorescence analysis was at least equal and possibly greater (in the range of 0·001–0·01 CFU).

Amplification and detection of clinical isolates

The PCR amplified product from fifteen isolates of *B. pertussis* and a negative control in presence of the beacon probe were analysed for fluorescence at 518 nm (Table 1). The fluorescence intensity of all the isolates were well above that of the control negative, indicating the presence of amplified *B. pertussis* target gene fragment in all the samples. The specific amplified DNA fragment (as illustrated in inset in Fig. 2) for each sample was also distinctly detectable by agarose gel electrophoretic analysis (not shown). Thus, the result obtained by spectrofluorometric method was in agreement with agarose gel analysis.

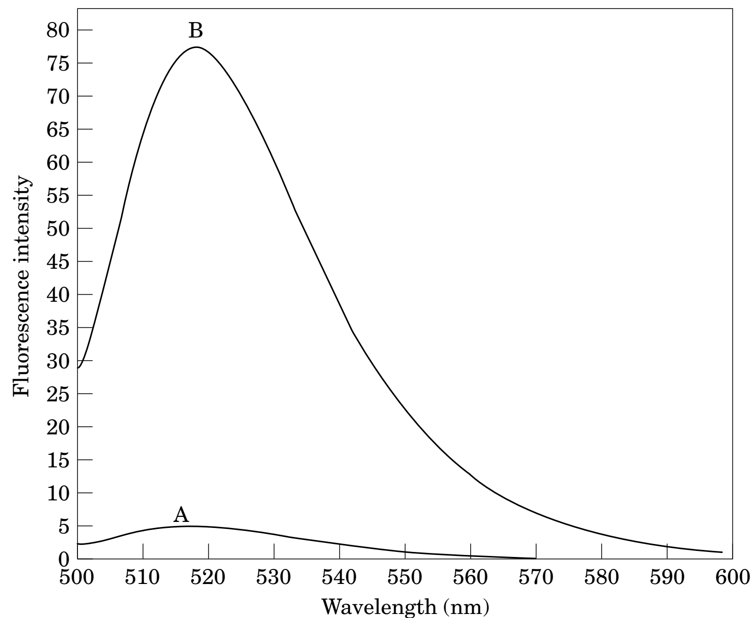

Fig. 1. Fluorescence emission spectrum analysis. 50 nM of probe were analysed for emission spectrum before hybridization (curve A) and after hybridization with 500 nM of complementary oligonucleotide (curve B), using excitation at 490 nm. The analysis was performed by reader model LS 50B (Perkin Elmer, Inc).

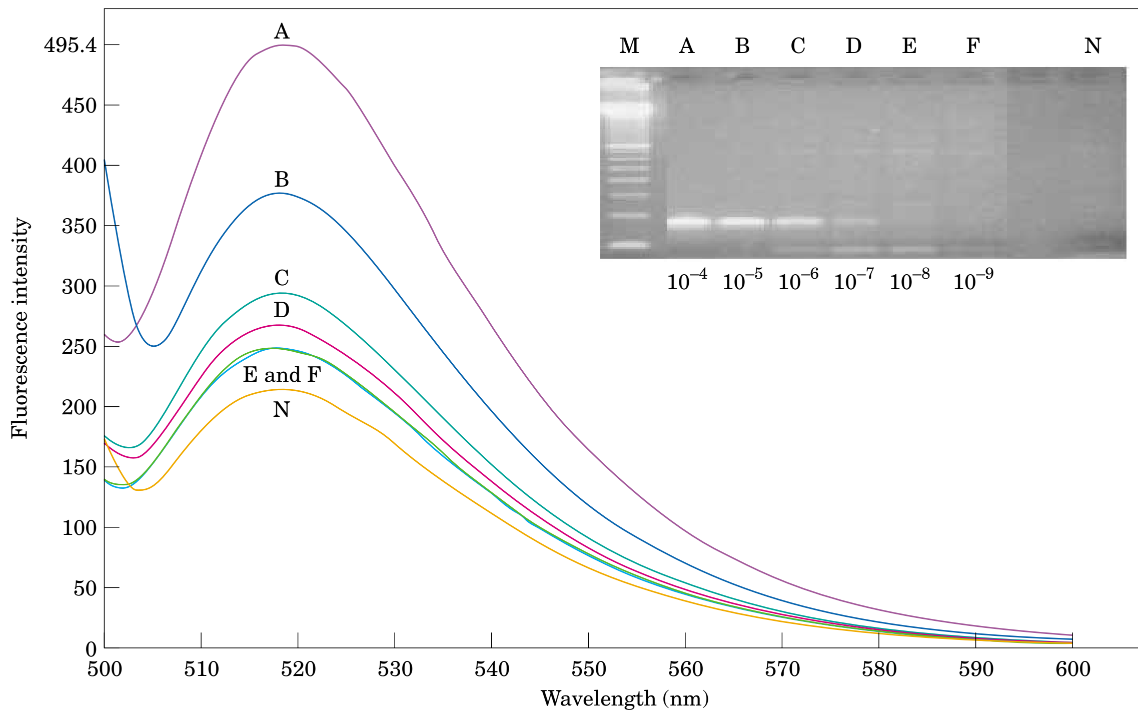

Fig. 2. Fluorescence analysis of the PCR products from serially diluted control samples. The dilution of sample was as indicated in each curve. The inset figure shows an agarose gel analysis of the PCR product. Dilution of sample was as shown at the bottom of each lane. Lane M, 100-bp molecular size marker (from New England Biolabs, Inc; Beberly, MA, USA). Lane N, control negative PCR.

DISCUSSION AND CONCLUSIONS

In the present study, *B. pertussis*-specific PCR products were detected by spectrofluorometry using homogeneous solution hybridization of a molecular beacon probe with PCR product. The use of the specific beacon probe in the spectrofluorometric protocol was validated in clinical isolates of *B. pertussis*.

In hybridization experiment of the beacon probe with an oligonucleotide unrelated to its target, fluor-

Table 1. Fluorescence analysis of clinical isolates of *B. pertussis* amplified in presence of the beacon probe

Sample number	51	52	53	54	60	63	68	69	71	72	78	81	105	488	9797
Increase in fluorescence above control negative (ΔF)	37·5	61·4	77·5	87·6	115	74·5	62·4	97·2	85·3	96·2	83·6	89·9	92·0	96·6	99·1

$\Delta F = (F_s - F_n)$, where F_s, and F_n were the fluorescence intensities of sample, and control negative respectively at 518 nm, with excitation at 490 nm. Each F_s value was average of three readings. Average of three F_n values was 481·2. ΔF value higher than 25 was indicative of presence of the target, and $\Delta F = (0 +/- 25)$ was considered absence of the target in a sample. Fluorescence measurements were performed using the multi-well microtitre plate attachment for reader model LS 50B.

escence remained the same as that of the probe only and with increased (>500 nM) addition of target oligonucleotide further increase (>18–20-fold) in fluorescence was not detected. This verified that all the probes were hybridized with the target (at the initial target concentration of 500 nM), the resulting increase in fluorescence occurred by specific hybridization and the signal to noise ratio was >18:1, for this probe preparation. The quality and performance competency of a beacon probe are determined by its signal to noise ratio and efficiency of quenching. The signal to noise ratio and the efficiency of quenching values of the probe used in the present study were comparable to those of the probes used in other studies.[10–12]

A recent study[12] demonstrated that binding of the amount of a beacon probe to its target strand and subsequent signal emission could be improved to a great extent by using an asymmetric PCR instead of a conventional symmetric PCR when the amplicon size was 307 bp. The size of the amplicon fragment in the present study was 153 bp, which is close to the sizes of amplicons of symmetric PCR detected by molecular beacon probes in previously published other reports.[11,13–15] Albeit there is no reported data, it is quite possible that beacon probe based detection is maximally efficient when the amplicon is of such short sizes. However, to support this notion further experiment addressing the issue of amplicon sizes and efficiency of beacon probe based detection will be needed.

In previous reports, primers used in PCR to detect *B. pertussis* have targeted one of the four chromosomal regions: (i) a region upstream of the porin gene,[3,16] (ii) pertussis toxin (PT) promotor region,[17,18] (iii) repeated insertion sequences,[7,19–24] and (iv) the adenylate cyclase toxin (ACT) gene.[25] PCR targeting the IS*481* repeated sequence were used to detect *B. pertussis* in recent studies[21–24] and there is evidence that these assays have a greater success rate of detection compared to culture[22] and are more sensitive than those using other gene targets.

However, sequencing data reported only very recently established that the genome of *Bordetella holmesii* contains regions homologous to the repeat sequence (IS*481*) of *B. pertussis* (personal communication, from Dr Udo Reischl, University of Regensburg, Regensburg, Germany). Therefore, PCR amplification of IS*481* repeat sequence region generated a positive signal for both *B. pertussis* and *B. holmesii* (ref #26 and unpublished observation in our laboratory). One approach to discriminate *B. pertussis* from *B. holmesii* in a clinical specimen, would include PCR targeting from another segment of the *B. pertussis* genome such as the PT promoter region[27] that experimentally has been found to exclude *B. holmesii*. This approach has the additional benefit of differentiating *Bordetella parapertussis*, which amplifies equally in amplification of PT promoter region but not the IS*481* with *B. pertussis*.[21,23] Therefore, the use of PCR assays targeting both the repeat sequence and PT promoter regions of *B. pertussis* would be complementary and provide specific detection of *B. pertussis*, *B. parapertussis* and *B. holmesii* in clinical specimens. Simultaneous amplification and detection of both target regions (PT promoter and IS*481*) of *B. pertussis* genome in a single PCR amplification is in development.

In view of the limitations of traditional diagnostic method, PCR-based procedures are being used ever increasingly in growing number of laboratories for detection of *B. pertussis*.[16–27] At present however, no FDA approved PCR test of *B. pertussis* is available commercially for adoption in reference laboratories, hospitals or small clinics. Different laboratories are developing and validating their own PCR test. Therefore, accuracy, sensitivity and specificity of PCR-tests of *B. pertussis* may vary from one laboratory to the other depending on the template-primer combination and particularly the detection system used in a PCR assay. Most PCR-detection systems use ethidium staining.[18,23–25] Nested PCR and detection by ethidium staining has been in use for its advantages regarding specificity and sensitivity.[23,27] However, carry over prevention system using uracil-*N*-glycosylase (UNG) that destroys the product from previous amplification can not be incorporated in a nested PCR. The use of carryover prevention is highly recommended for

PCR-based diagnostics of *B. pertussis*.[28] Several other detection systems that provide increased sensitivity and specificity are also in use for detection of *B. pertussis*, these include multi-step involving Southern blotting, dot blotting, liquid hybridization and hybridization of post-PCR products with labeled oligonucleotide probes.[20,23,25]

The present molecular-beacon-based method offers a homogeneous solution phase hybridization assay in which amplification and detection of fluorescent signal emitted from the beacon probe hybridized to the amplified DNA of *B. pertussis* can occur in one tube. This makes the assay method easy to perform, and less time consuming by reducing the number of steps compared to other probing methods.[7,16,23,25] The risk of carry-over contamination is also considerably minimized by the advantage of performing the entire assay in a closed tube or microtiter plate. Therefore, incorporation of UNG may not be necessary in beacon probe-based detection. This makes the beacon probe based detection of *B. pertussis* more advantageous to adopt in clinical diagnostic laboratories as compared to other sensitive detection procedures of PCR product. However like any other molecular methods, beacon probe based detection of *B. pertussis* in many clinical diagnostic laboratories such as those in hospitals, and small clinics may be quite technically demanding, since appropriately trained technical personal may not be there to characterize the probe and standardize the assay. It may also be unaffordable because of the present time cost in commercial synthesis of the beacon probe, its purification and in the necessary instrumentation. Whereas large diagnostic laboratories or high throughput reference laboratories with trained personnel may find it economical and advantageous in view of its increased sensitivity, rapidity and simplicity in assay performance.

ACKNOWLEDGEMENTS

This research has been conducted in compliance with all applicable Federal Regulations governing the protection of human subjects in research. This represents report no. 00–46, supported by Department of Defense's Global Emerging Infections System and Bureau of Medicine and Surgery, Department of the Navy, Washington, DC under work unit no. 6609 Navy Environmental Health Center N6854699WR00028 reimbursable. The views expressed in this article are those of the authors and do not reflect the official policy or position of the Department of the Navy, Department of Defense, or the US Government. Approved for public release; distribution unlimited.

REFERENCES

1. Brennan, M., Strebel, P., George, H., Yih, W. K., Tachdjian, R., Lett, S. M., *et al.* (2000). Evidence for transmission of pertussis in schools, Massachusetts, 1996: epidemiologic data supported by pulsed-field gel electrophoresis studies. *Journal of Infectious Disease* **181**, 210–5.
2. Robbins, J. B. (1999). *Pertussis* in adults: introduction. *Clinical Infectious Disease* **28**, S91–3.
3. Jansen, D. L., Gray, G. C., Putnam, S. D., Lynn, F. & Meade, B. D. (1997). Evaluation of *Pertussis* in US. marine corps trainees. *Clinical Infectious Disease* **25**, 1099–107.
4. Cherry, J. D. (1999). Epidemiological, clinical and laboratory aspects of *pertussis* in adults. *Clinical Infectious Disease* **28**, S112–7.
5. Müller, F. C., Hoppe, J. E. & Wirsing von König, C. H. (1997). Laboratory diagnosis of *pertussis*: State of the Art in 1997. *Journal of Clinical Microbiology* **35**, 2435–43.
6. Matlow, A. G., Nelson, S., Wray, R. & Cox, P. (1997). Nosocomial acquisition of pertussis diagnosed by polymerase chain reaction. *Infection Control and Hospital Epidemiology* **18**, 715–6.
7. Glare, E. M., Paton, J. C., Premier, R. R., Lawrence, A. J. & Nisbet, I. T. (1990). Analysis of repetitive DNA sequence from *Bordetella pertussis* and its application to the diagnosis of pertussis using the polymerase chain reaction. *Journal of Clinical. Microbiology* **28**, 1982–7.
8. Poddar, S. K., Sawyer, M. H. & Connor, J. D. (1998). 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. *Journal of Clinical Laboratory Analysis* **12**, 238–41.
9. Poddar, S. K., Sawyer, M. H. & Connor, J. D. (1998). Effect of inhibitors in clinical specimens on Taq and Tth DNA polymerase-based PCR amplification of influenza A virus. *Journal of Medical Microbiology* **47**, 1131–5.
10. Poddar, S. K. (1999). Detection of adenovirus using PCR and molecular beacon. *Journal of Virological Methods* **82**, 19–26.
11. Tyagi, S., Bratu, D. P. & Kramer, F. R. (1998). Multicolor molecular beacons for allele discrimination. *Nature Biotechnology* **16**, 49–53.
12. Poddar, S. K. (2000). Symmetric vs asymmetric PCR and molecular beacon probe in the detection of a target gene of adenovirus. *Molecular and Cellular Probes* **14**, 25–32.
13. Piatek, A. S., Tyagi, S., Pol, A. C., Telenti, A., Miller, L. P., Kramer, F. R. & Alland, D. (1998). Molecular beacon sequence analysis for detecting drug resistance in *Mycobacterium tuberculosis*. *Nature Biotechnology* **16**, 359–63.
14. Gionata, L., Schijndel, H., Gemen, B., Kramer, F. R. & Schoen, C. D. (1998). Molecular beacon probes combined with amplification by NASBA enable homogeneous, real-time detection of RNA. *Nucleic Acids Research* **26**, 2150–5.
15. Vet, J. A. M., Majithia, A. R., Marras, S. A. E., Tyagi, S., Dube, S., Poiesz, B. J. & Krammer, F. R. (1999). Multiplex detection of four pathogenic retroviruses

using molecular beacons. *Proceedings of National Academy of Science, USA* **96**, 6394–9.

16. Li, Z. M., Jansen, D. L., Finn, T. M., Halperin, S. A., Kasina, A., O'Connor, S. P., *et al*. (1994). Identification of *Bordetella pertussis* infections by shared-primer PCR. *Journal of Clinical Microbiology* **32**, 783–9.
17. Stefanelli, P., Giuliano, M., Spigaglia, P. & Mastrantonio, P. (1996). Polymerase chain reaction for the identification of *Bordetella pertussis* and *Bordetella parapertussis*. *Diagnostic Microbiology of Infectious Disease* **24**, 197–200.
18. Mastrantonio, P., Stefanelli, P. & Giuliano, M. (1996). Polymerase chain reaction for the detection of *Bordetella pertussis* in clinical nasopharyngeal aspirates. *Journal of Medical Microbiology* **44**, 201–66.
19. Edelman, K., Nikkari, S., Ruuskanen, O., He, Q., Viljanen, M. & Mertsola, J. (1996). Detection of *Bordetella pertussis* by polymerase chain reaction and culture in the nasopharynx of erythromycin-treated infants with *pertussis*. *Pediatric Infectious Diseases Journal* **15**, 54–7.
20. Nelson, S., Matlow, A., McDowell, C., Roscoe, M., Karmali, M., Penn, L. & Dyster, L. (1997). Detection of *Bordetella pertussis* in clinical specimens by PCR and a microtiter plate-based DNA hybridization assay. *Journal of Clinical Microbiology* **35**, 117–20.
21. Lind-brandberg, L., Welinder-Olsson, C., Lagergard, T., Taranger, J., Trollfors, B. & Zackrisson, G. (1998). Evaluation of PCR for diagnosis of *Bordetella pertussis* and *Bordetella parapertussis* infections. *Journal of Clinical Microbiology* **36**, 679–83.
22. Erlandsson, A., Backman, A., Tornqvist, E. & Olsen, P. (1997). PCR assay or culture for diagnosis of *Bordetella pertussis* in the routine diagnostic laboratory? *Journal of Infection* **35**, 221–4.
23. Farrell, D. J., Daggard, G. & Mukkur, T. K. S. (1999). Nested duplex PCR to detect *Bordetella pertussis* and *Bordetella parapertussis* and its application in diagnosis of pertussis in nonmetropolitan southeast queenland, Australia. *Journal of Clinical Microbiology* **37**, 606–10.
24. Loeffelholz, M. J., Thompson, C. J., Long, K. S. & Gilchrist, M. J. R. (1999). Comparison of PCR, culture and direct fluorescent-antibody testing for detection of *Bordetella pertussis*. *Journal of Clinical Microbiology* **37**, 2872–6.
25. Douglas, E., Coole, J. G., Parton, R. & McPheat, W. (1993). Identification of *Bordetella pertussis* in nasopharyngeal swabs by PCR amplification of a region of the adenylate cyclase gene. *Journal of Medical Microbiology* **38**, 140–4.
26. Loeffelholz, M. J., Thompson, C. J., Long, K. S. & Gilchrist, M. J. R. (2000). Detection of *Bordetella holmesii* using *Bordetella pertussis* IS481 PCR assay. *Journal of Clinical Microbiology* **38**, 467.
27. Reizenstein, E., Lindberg, L., Mollby, R. & Hallander, H. O. (1996). Validation of nested Bordetella PCR in pertussis vaccine trial. *Journal of Clinical Microbiology* **34**, 810–5.
28. Meade, B. D. & Bollen, A. (1994). Recommendations for the use of the polymerase chain reaction in the diagnosis of *Bordetella pertussis* infections. *Journal of Medical Microbiology* **41**, 51–5.