Development and Use of an Internal Positive Control for Detection of *Bordetella pertussis* by PCR

Stéphanie Herwegh,¹ Christophe Carnoy,^{2,3} Frédéric Wallet,¹ Caroline Loïez,¹
and René J. Courcol^{1*}

Laboratoire de Bactériologie-Hygiène, Centre Hospitalier Régional Universitaire, Lille, France,¹ INSERM E0364,
Institut de Biologie de Lille, 1 rue du Pr. Calmette, 59021 Lille Cedex, France,² and Faculté des
Sciences Pharmaceutiques et Biologiques, Lille, France³

Received 18 August 2004/Returned for modification 19 August 2004/Accepted 20 December 2004

An internal control of amplification was constructed by recombinant PCR to detect PCR inhibitors. This exogenous DNA was included in the reaction mixture and coamplified with the target gene. This detection was successfully applied to the diagnosis of whooping cough by amplification of a fragment of *Bordetella pertussis* IS481.

The detection of inhibitors of amplification remains a major problem which can partly be solved by the use of internal controls (ICs) of amplification (5, 6, 19). In addition to some commercially available systems, inhibitors can be detected either by spiking negative samples with the target DNA (7), by diluting the specimen to minimize the effects of inhibitors (16), or by adding a control template (2, 10, 15).

In this study, we developed for diagnosis of *Bordetella pertussis* a method to detect PCR inhibitors in clinical samples. We constructed an IC that can be amplified by the same primer pair as wild-type target DNA. IC was obtained by insertion of an exogenous DNA fragment into reference strain target by recombinant PCR (1, 3, 4).

B. pertussis Tohama phase I strain was the reference strain used to amplify the repetitive insertion sequence IS481. *Escherichia coli* DH5 α was used as host for recombinant vector pMos (Amersham Pharmacia Biotech, Raiham, England). Amplified DNA from the *B. pertussis* reference strain was purified with a QIAquick PCR purification kit (QIAGEN, Hilden, Germany). This wild-type fragment was then directly used as template for recombinant PCR.

A 191-bp fragment from pertussis toxin gene amplified with primers Pt1 (5'-CCAACGCGCATGCGTGAGATTGCTC-3') and Pt2 (5'-CCCTCTGCGTTTTGATGGTGCCTATTTT A-3') was introduced into the wild-type IS481 DNA (203 bp) by recombinant PCR (Fig. 1). Two internal primers, harboring a 5' end complementarity to the extremities of the exogenous toxin gene fragment to be inserted, were designed. In a first step, two PCR fragments were independently generated: fragment A with external primer A (Bp13, 5'-CCGCGCTGTGC CATGAGCTGG-3') and internal primer A (Pt1rv/Bp15, 5'-G ACGAATCTGCACGCATGCGCGTTGGGGGCCCGCA AGGCCGACTGGATGAAGCGTTTCG-3') and fragment B with external primer B (Bp14, 5'-GATGCCTTGGTGGGGT CGATG-3') and internal primer B (Pt2rv/Bp16, 5'-TAAAT

AGGCACCATCAAAACGCAGAGGGGGGCCCTGAGTG GGCTTACGCTCACACCTACCA-3'). These two amplicons and the 191-bp fragment were submitted to a second step of extension without primers. The third step consisted of a PCR with Bp13 and Bp14 in order to amplify the future internal control (Fig. 1). The resulting fragment (406 bp) was cloned into pMos vector. Recombinant plasmid was designated pSIBp. Construction was confirmed by PCR on thermolysates of *E. coli* strains containing the recombinant plasmid. Two ApaI sites flanking the exogenous DNA were present on Pt1rv/Bp15 and Pt2rv/Bp16 and were used to check the construction by ApaI restriction of the fragment amplified from an *E. coli* thermolysate. Three fragments of 100, 105, and 201 bp were obtained as expected. One clone of *E. coli* containing the correct recombinant plasmid was kept at -80°C. pSIBp plasmid was used as IC for detection in clinical samples.

IC concentration was optimized by adding various quantities of IC to PCRs containing constant amounts of wild-type DNA. Tenfold dilutions of the pSIBp plasmid were made, from 10 ng/ μ l to 10⁻¹⁰ ng/ μ l, and 5 μ l was added to amplification reactions containing a constant amount of bacterial DNA known to be the threshold previously determined (10³ CFU/ml). A 10⁻⁷ dilution of the pSIBp was determined as the optimal amount of IC. This dilution of plasmid corresponds to 10⁴ molecules of internal standard versus 10 copies of the bacterial genome.

At high bacterial inoculums, only the wild-type fragment was amplified and no internal standard was detected (Fig. 2). Wild-type DNA is preferentially amplified because of its shorter length and because competition is minimized by addition of a small amount of IC. Since an excess of IC or genomic DNA may inhibit the amplification of the other by competition (Fig. 2), it is necessary to optimize the quantity of IC DNA added into the reaction mixtures. The amount of IC added in each reaction tube may seem quite high (10⁴ plasmid copies) but is in accordance with previously published results (11).

Specimens for *B. pertussis* detection were nasopharyngeal aspirations (NPA) routinely received by the Bacteriology Laboratory of the Lille University Medical Center, France. The NPA collection tubing was flushed out by aspiration of 1 ml of

* Corresponding author. Mailing address: Laboratoire de Bactériologie-Hygiène, Hôpital A. Calmette, F-59037-Lille Cedex, France. Phone: 33-320.444.908. Fax: 33-320.444.895. E-mail: rcourcol@chru-lille.fr.

FIG. 1. Construction of the internal control by recombinant PCR. Internal primers harboring a 5' complementarity to the extremities of pertussis toxin fragment were used for insertion into IS481 amplification product, leading to the obtention of a 406-bp internal control.

sterile distilled water. Culture and identification of *B. pertussis* were performed according to conventional methods (13). For PCR detection, the NPA suspensions were boiled for 15 min and then centrifuged. Five microliters of the supernatant was directly used for amplification of IS481. Reaction mixtures were subjected to 35 cycles of amplification (30 s at 94°C, 30 s at 55°C, and 60 s at 72°C) (20). Amplicons were revealed by agarose gel electrophoresis followed by ethidium bromide staining.

One hundred twenty clinical samples were used to test the reliability of the *B. pertussis* IS481 IC. After a first amplification with pure NPA lysates, 33 samples (27.5%) were IS481 positive and 44 specimens (36.6%) were negative for IS481 but positive for the IC; in 43 samples (35.8%) neither the IC nor IS481 were detectable, suggesting the presence of inhibitors in the NPA samples. A second PCR assay was performed on these 43 NPA lysates, which were diluted 10-fold. Three specimens were IS481 positive, 25 specimens (20.8%) were negative for IS481 but positive for the IC, and 15 (12.5%) specimens remained inhibitory to the amplification of both IS481 and the IC. Following development tests, a 10-fold dilution of the ther-

molysate was included in our protocol for the diagnosis of *B. pertussis*.

A retrospective study over 4 years on 1,188 NPA specimens to detect *B. pertussis* by PCR was performed. The detection of IS481 was positive in 111 cases (9.3%), whereas 200 samples (16.8%) were negative for IS481 and the IC when tested with pure lysates but positive for the IC with 10-fold-diluted samples, 821 samples were negative for IS481 but positive for the IC, and 56 samples (4.7%) were negative for both IS481 and the IC. For the latter samples, PCRs obtained with DNA extracted with a commercial kit were still negative for IS481 but positive for IC. Although some authors (9, 14) have described no inhibition phenomenon within their clinical specimens, inhibition rates from 5 to 42% are quite usual (8, 16–18), depending on the nature of the clinical samples and the DNA extraction method. The present results, established on a large number of specimens, gave a smaller number of inhibiting specimens (21.5%) than the development study (35.8%) and were in agreement with those reported in the literature.

As the IC was amplified by the same primer pair used for the detection of the microorganism, there were no major modifications of the amplification protocols. Moreover, since the diagnosis of *B. pertussis* by IS481 was usually confirmed by a second PCR targeting pertussis toxin, our standardized IC, combining IS481 and pertussis toxin, was used as positive control for both assays. It should be possible to improve the amplification methods by addition of the IC into the clinical specimen prior to extraction (12). This IC could allow simultaneous detection of inefficient sample preparations.

We are very grateful to A. Devalckenaere and S. Armand for their technical assistance.

REFERENCES

- Donnenberg, M. S., J. Yu, and J. B. Kaper. 1993. A second chromosomal gene necessary for intimate attachment of enteropathogenic *Escherichia coli* to epithelial cells. *J. Bacteriol.* 175:4670–4680.
- Gibb, A. P., and S. Wong. 1998. Inhibition of PCR by agar from bacteriological transport media. *J. Clin. Microbiol.* 36:275–276.
- Higushi, R. 1990. Recombinant PCR, p. 177–183. In M. Innis (ed.), *PCR protocols: a guide to methods and applications*. Academic Press, Inc., San Diego, Calif.
- Ho, S. N., H. D. Hunt, R. M. Horton, J. K. Pullen, and L. Pease. 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene* 77:51–59.
- Hoorfar, J., N. Cook, B. Malorny, M. Wagner, D. de Medici, A. Abdulmajid, and P. Fach. 2003. Making internal amplification control mandatory for diagnostic PCR. *J. Clin. Microbiol.* 41:5835.
- Hoorfar, J., B. Malorny, A. Abdulmajid, N. Cook, M. Wagner, and P. Fach. 2004. Practical considerations in design of internal amplification controls for diagnostic PCR assays. *J. Clin. Microbiol.* 42:1863–1868.
- Jantos, C. A., R. Roggendorf, F. N. Wuppermann, and J. H. Hegemann. 1998. Rapid detection of *Chlamydia pneumoniae* by PCR-enzyme immunoassay. *J. Clin. Microbiol.* 36:1890–1894.
- Kolk, A. H. J., G. T. Noordhoek, O. De Leeuw, S. Kuijper, and J. D. A. Van Embden. 1994. *Mycobacterium smegmatis* strain for detection of *Mycobacterium tuberculosis* by PCR used as internal control for inhibition of amplification and for quantitation of bacteria. *J. Clin. Microbiol.* 32:1354–1356.
- Lind-Brandberg, L., C. Welinder-Olsson, T. Lagergard, J. Taranger, B. Trollfors, and G. Zackrisson. 1998. Evaluation of PCR for diagnosis of *Bordetella pertussis* and *Bordetella parapertussis* infections. *J. Clin. Microbiol.* 36:679–683.
- Martineau, F., F. J. Picard, P. H. Roy, M. Ouellette, and M. G. Bergeron. 1998. Species-specific and ubiquitous-DNA-based assays for rapid identification of *Staphylococcus aureus*. *J. Clin. Microbiol.* 36:618–623.
- Mathis, A., and P. Deplazes. 1995. PCR and in vitro cultivation for detection of *Leishmania* spp. in diagnostic samples from humans and dogs. *J. Clin. Microbiol.* 33:1145–1149.
- Morré, S. A., P. Sillekens, M. V. Jacobs, P. van Aarle, S. de Blok, B. van Gemen, J. M. Walboomers, C. J. L. M. Meijer, and A. J. van den Brule. 1996.

FIG. 2. Competition between IS481 (203 bp) and internal control (406 bp). A constant amount of IC (10^4 plasmid copies) was coamplified with various *B. pertussis* Tohama inoculums: 10^7 CFU/ml (lane 1), 10^6 CFU/ml (lane 2), 10^5 CFU/ml (lane 3), 10^4 CFU/ml (lane 4), 10^3 CFU/ml (lane 5), 10^2 CFU/ml (lane 6), and 100-bp ladder (lane M).

- RNA amplification by nucleic acid sequence-based amplification with an internal standard enables reliable detection of *Chlamydia trachomatis* in cervical scrapings and urine samples. J. Clin. Microbiol. **34**:3108–3114.
13. Murray, P. R., E. J. Baron, M. A. Pfaller, F. C. Tenover, and R. H. Tenover (ed.). 1999. Manual of clinical microbiology, 7th ed. ASM Press, Washington, D.C.
  14. Nelson, S., A. Matlow, C. McDowell, M. Roscoe, M. Karmali, L. Penn, and L. Dyster. 1997. Detection of *Bordetella pertussis* in clinical specimens by PCR and microtiter plate-based DNA hybridization assay. J. Clin. Microbiol. **35**:117–120.
  15. Saiki, R. K., S. Scharf, F. Faloona, K. B. Mullis, G. T. Horn, H. A. Erlich, and N. Arnheim. 1985. Enzymatic amplification of β -globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science **230**:1350–1354.
  16. Thoreson, A. C. E., M. Borre, L. P. Andersen, F. Jorgensen, S. Kiilerich, J. Scheidel, J. Rath, and K. A. Krogfelt. 1999. *Helicobacter pylori* detection in human biopsies: a competitive PCR assay with internal control reveals false results. FEMS Immunol. Med. Microbiol. **24**:201–208.
  17. Tjhe, T. J., F. J. van Kuppeveld, R. Roosendaal, W. J. Melchers, R. Gordijn, D. M. MacLaren, J. M. Walboomers, C. J. Meijer, and A. J. van den Brule. 1994. Direct PCR enables detection of *Mycoplasma pneumoniae* in patients with respiratory tract infections. J. Clin. Microbiol. **32**:11–16.
  18. Ursi, J. P., D. Ursi, M. Ieven, and S. R. Pattyn. 1992. Utility of an internal control for the polymerase chain reaction. Application to detection of *Mycoplasma pneumoniae* in clinical specimens. APMIS **100**:635–639.
  19. Wadowsky, R. M., S. Laus, T. Libert, S. J. States, and G. D. Ehrlich. 1994. Inhibition of PCR-based assay for *Bordetella pertussis* by using calcium alginate fiber and aluminum shaft components of a nasopharyngeal swab. J. Clin. Microbiol. **32**:1054–1057.
  20. Whelen, A. C., and D. H. Persing. 1996. The role of nucleic acid amplification and detection in the clinical microbiology laboratory. Annu. Rev. Microbiol. **50**:349–373.