

## Polymerase Chain Reaction Assay for Pertussis: Simultaneous Detection and Discrimination of *Bordetella pertussis* and *Bordetella parapertussis*

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**A polymerase chain reaction (PCR) assay which allows the simultaneous detection and discrimination of the two causative agents of pertussis, *Bordetella pertussis* and *Bordetella parapertussis*, was developed. Primer pairs were based on insertion sequence elements IS481 and IS1001. IS481 is specific for *B. pertussis* and is present in about 80 copies per cell, while IS1001 is specific for *B. parapertussis* and is found in 20 copies per cell. An internal control was included in the PCR assay to monitor the performance of the PCR and to identify possible inhibitory components in clinical samples. Discrimination of amplified DNA derived from the internal control, *B. pertussis*, or *B. parapertussis* was accomplished by differential spacing of the primers. The sensitivity of the combined PCR method was found to be very high and allowed the detection of one cell of either pathogen. The usefulness of the method was investigated by using a limited number of clinical samples derived from patients with serologically proven pertussis.**

Whooping cough, or pertussis, is a highly contagious respiratory disease caused by two *Bordetella* species, *B. pertussis* and *B. parapertussis*. At present, estimation of the incidence of *Bordetella* infection among humans is based on clinical diagnosis, serology, and/or culture. An analysis based on clinical diagnosis will underestimate the incidence, because a large fraction of infections probably do not elicit symptoms (8, 9). In the Netherlands, notification of pertussis is based on clinical diagnosis which has been confirmed by culture or serology. Confirmation by culture is problematic in view of the difficulty in culturing *B. pertussis* from humans; depending on the stage of the disease, the successful isolation rate varies between 60 (early in disease) and 6% (late in disease) (13). The interpretation of serology is complicated by the fact that the observed titers may be the result of vaccination or of current and/or previous infections. Diagnosis based on serology has been improved recently by analyzing paired sera taken from a single patient at different time points (16). Thus, one can now observe an increase in antibody titer which can be due only to a current infection. However, a serious disadvantage of the analysis of paired serum samples is that it is cumbersome for the patient and physician involved; this may result in a decrease in notification. Indeed, in the Netherlands a decrease in notification has been observed following the introduction of diagnoses based on paired sera.

An additional drawback of the present methods used for surveillance of pertussis is that they may underestimate the incidence of *B. parapertussis*. It is assumed that a minority of pertussis cases (3 to 35%) is caused by *B. parapertussis* (7, 12), and usually milder symptoms are observed. Although *B. parapertussis* is easier to grow than *B. pertussis*, the occurrence of less-severe symptoms can lead to a situation in which *B. parapertussis* infections are overlooked. Another

factor which may result in an apparently low incidence of *B. parapertussis* infections is the fact that antigens used for serological diagnosis are derived from *B. pertussis*. Since both species harbor many different surface antigens, present methods used for serological surveillance will be biased toward *B. pertussis* (unpublished data).

Clearly, there is room for improvement of the present surveillance methods for pertussis. This improvement is especially pertinent in view of the evaluation of the new generation of acellular pertussis vaccines (17). The acellular vaccines will be composed of one to four antigens derived from *B. pertussis* and may confer insufficient protection against *B. parapertussis*, since the two species show many differences in surface antigens (1, 14). This conjecture is substantiated by experiments with a mouse model which revealed that no cross-protection was observed between antigens derived from *B. pertussis* and from *B. parapertussis* (6).

Detection of *Bordetella* based on the polymerase chain reaction (PCR) seemed the most promising approach to us, because this method in principle allows the detection of a single bacterium. A first step in the development of PCR-based detection is the identification of DNA sequences specific for *B. pertussis* or *B. parapertussis*. Repeated DNA sequences are especially suitable targets for PCR, since they enhance the sensitivity of the detection relative to single-copy sequences. A repeated DNA sequence, designated IS481, specific for *B. pertussis* has been described elsewhere (10, 11) and has been used successfully for the detection of *B. pertussis* by PCR (4, 5). Recently, we have identified a repeated sequence, designated IS1001, that is specific for *B. parapertussis* and *B. bronchiseptica* (18). Thus, it seemed feasible to design a PCR-based diagnostic test with which it is possible to detect both *B. pertussis* and *B. parapertussis* and, moreover, to discriminate between the two pathogens. This work describes such a test and its performance in the analysis of a limited number of clinical samples.

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TABLE 1. Bacterial strains used in this study

Species	Strain
<i>Bacillus cereus</i> .....	8900855
<i>Bacteroides fragilis</i> .....	881065
<i>Bordetella pertussis</i> .....	Tohama
<i>Bordetella parapertussis</i> .....	B24
<i>Branhamella catarrhalis</i> .....	873640
<i>Corynebacterium bovis</i> .....	890076
<i>Escherichia coli</i> .....	W3110
<i>Haemophilus influenzae</i> .....	8901300
<i>Haemophilus parainfluenzae</i> .....	891369
<i>Klebsiella pneumoniae</i> .....	900245
<i>Legionella pneumophila</i> .....	1-serogr. 1
<i>Moraxella nonliquefaciens</i> .....	891371
<i>Neisseria meningitidis</i> .....	890783
<i>Pasteurella multocida</i> .....	890772
<i>Pseudomonas aeruginosa</i> .....	B92556
<i>Staphylococcus aureus</i> .....	891248
<i>Staphylococcus epidermidis</i> .....	891450
<i>Streptococcus pneumoniae</i> .....	91193
<i>Streptococcus pyogenes</i> .....	B91191
<i>Streptococcus salivarius</i> .....	A91191
<i>Streptococcus agalactiae</i> .....	B91197

MATERIALS AND METHODS

**Bacterial strains and plasmids.** The bacterial strains used in this study are listed in Table 1. Plasmid pRPP6 was constructed by inserting a 600-bp *HpaI*-*ClaI* IS481 fragment derived from cosmid p11-11 (10) into the *HpaI*-*ClaI* sites of pBluescript (Stratagene, La Jolla, Calif.) (Fig. 1). The cosmid p11-11 was kindly provided by Alison Weiss. Plasmid pRPP10 was constructed by inserting an 800-bp *PstI* fragment, derived from pRPP1 (18), into the *PstI* site of PEMBL19 (3). The 800-bp *PstI* fragment contains mainly IS1001 sequences (Fig. 1).

**PCR.** PCR amplifications were performed in a final volume of 25 µl containing 67 mM Tris-HCl (pH 8.8), 16.6 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 6.7 mM MgCl<sub>2</sub>, 10 mM β-mercaptoethanol, 5% dimethyl sulfoxide, 200 µM each deoxynucleotide, 125 ng of

each primer (initially), and 0.6 U of AmpliTaq polymerase (Perkin-Elmer Cetus). The buffer was modified from that described by Houard et al. (5). The reaction mixtures were preheated at 94°C for 3 min in a DNA thermocycler (Perkin-Elmer Cetus), and subsequently 33 amplification cycles of 1 min at 94°C, 1.5 min at 60°C, and 2 min at 72°C, followed by 5 min at 72°C, were performed to complete the final PCR.

**Sample preparation to optimize the PCR assay.** *B. pertussis* and *B. parapertussis* were grown on Bordet-Gengou plates for 3 days at 35°C. The following methods for DNA preparation were compared to optimize the PCR assay. For method A, dilutions of *B. pertussis* cells were suspended in 50 µl of lysis buffer (50 mM Tris-HCl pH 8.5, 50 mM NaCl, 4 mM MgCl<sub>2</sub>, 2 mM dithiothreitol, 0.45% Nonidet P-40, 100 µg of proteinase K per ml, 100 µg of herring sperm DNA per ml). After 2 to 3 h of incubation at 55°C, the samples were heated at 100°C for 10 min to inactivate the proteinase K. After ethanol precipitation, DNA was subjected directly to PCR. Method B was the same as method A, except that after heating at 100°C, DNA from lysed bacteria was recovered by adsorption to diatoms in the presence of guanidinium thiocyanate as has been described by Boom et al. (2). For method C, cells were suspended in H<sub>2</sub>O and lysed by incubation at 100°C for 15 min. For methods D and E, cells were suspended in physiological salt (method D) or physiological salt supplemented with 1 mM EDTA (PE; method E). In all methods, serial dilutions of bacterial cells, ranging from 0.2 × 10<sup>6</sup> to 0.2 × 10<sup>2</sup>, were made, of which 5 µl was used for the PCR. The numbers of cells were assessed by measuring the optical density at 600 nm. A suspension of *B. pertussis* with an optical density at 600 nm of 1 was found to contain 2 × 10<sup>9</sup> CFU per ml.

**Standard PCR procedure for clinical samples.** Clinical specimens from children suspected to be infected with *B. pertussis* or *B. parapertussis* were collected as nasopharyngeal swabs (dacron; Medical Wire, Cosham, United Kingdom). The nasopharyngeal swabs were transported dry and suspended in 200 µl of PE. Samples were heated for 15 min at 100°C to lyse the cells, and 5 µl of each sample was used

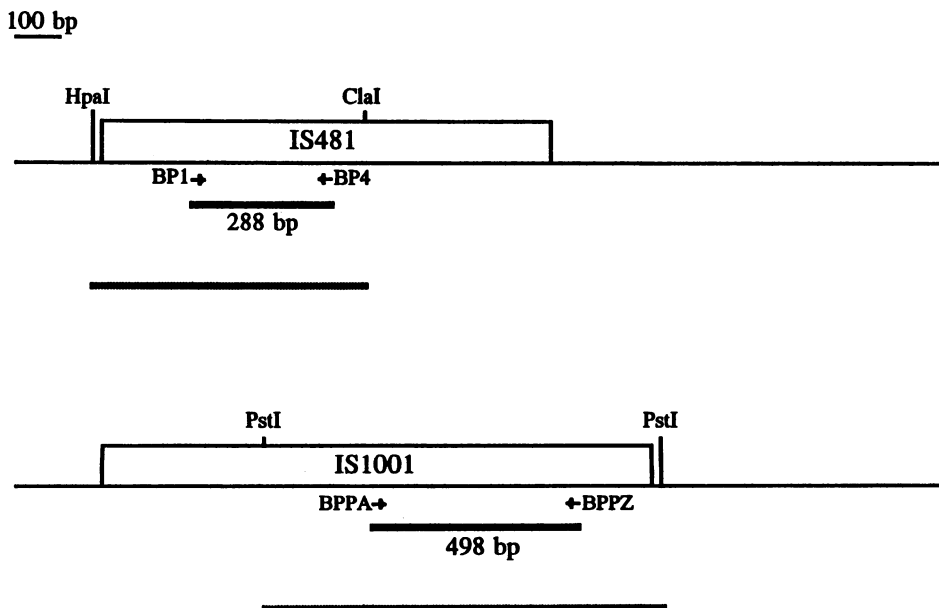


FIG. 1. Schematic representation of the *B. pertussis* and *B. parapertussis* insertion sequence elements IS481 and IS1001, respectively. The primers used for the PCR are indicated by arrows, and the amplified DNA products are indicated by black bars. The lengths of the amplified products are indicated below the bars. The dashed lines represent the restriction fragments that were used as probes on clinical samples.

for amplification reactions. The reaction mixture (25  $\mu$ l) contained 30 ng of each primer BP1 and BP4, 120 ng of each primer BPPA and BPPZ, and 30 fg of pRPP100, which served as an internal control for the polymerization reaction. Amplification was performed with 1.2 U of AmpliTaq polymerase. PCR conditions and the PCR mix were used as described above. Samples in which the 150-bp internal control fragment was not detected were treated as follows. The sample was extracted with an equal volume of phenol-chloroform (1:1), precipitated with ethanol, and suspended in one-seventh of its original volume. Subsequently, 5  $\mu$ l was used for PCR.

**Primers.** On the basis of the sequence of IS481 as published by McLafferty et al. (10), two oligonucleotides were synthesized by using an Applied Biosystems DNA synthesizer (Applied Biosystems, Inc., Foster City, Calif.). Oligonucleotides BP1 (5'-GGGGTCACCGCGCCGACTGT-3') and BP4 (5'-GGGCCTGATGCTCGTGATAGCGC-3') correspond to bp 208 to 228 and 476 to 496 of IS481. Oligonucleotides BPPA (5'-CGCCGCTTGATGACCTTGATA-3') and BPPZ (5'-CACCGCCTACGAGTTGGAGAT-3') are based on the sequence of IS1001 (18) and correspond to bp 1211 to 1232 and bp 734 to 755, respectively. The oligonucleotides were used without further purification.

**Construction of the internal control of the PCR.** A DNA fragment to be used as an internal control for the PCR was generated by amplification of *Escherichia coli* DNA, with *B. pertussis*- and *B. parapertussis*-specific primers BP1 and BPPZ, respectively. DNA fragments were generated by annealing the primers at low stringency; 33 amplification cycles of 1 min at 94°C, 1.5 min at 40°C (primer annealing), and 2 min at 72°C, followed by 5 min at 72°C, were performed to complete the final PCR. After PCR, approximately 15 fragments were generated, and a number of fragments of suitable size (i.e., different from those of the *B. pertussis*- and *B. parapertussis*-specific amplification products) were isolated from the gel with GeneClean (Bio 101, La Jolla, Calif.). After treatment with Klenow DNA polymerase (1 U, 1 mM deoxynucleotide triphosphate mixture, 20 min at 30°C) to create blunt ends, the fragments were inserted into pUC21 (Boehringer GmbH, Mannheim, Germany), and their flanking DNA regions were sequenced. One plasmid, designated pRPP100 and harboring a 150-bp DNA fragment flanked by primers BP1 and BPPZ, was selected.

**Labelling of DNA probes.** To obtain probes specific for IS481 and IS1001, the plasmids pRPP6 and pRPP10 were cleaved with *Cla*I-*Hpa*I and *Pst*I, respectively (Fig. 1). Subsequently, the 600-bp *Cla*I-*Hpa*I and 800-bp *Pst*I DNA fragments were purified by gel electrophoresis and extracted from the agarose with GeneClean (Bio 101). Finally, 100 ng of DNA was labelled with digoxigenin, according to the instructions of the AMPPD kit (Boehringer).

**Analysis of PCR products.** After the PCR, six  $\mu$ l of the reaction mixture was analyzed by gel electrophoresis in 2% agarose gels containing 500 ng of ethidium bromide per ml. The results of the PCR were judged after visualization of DNA fragments by UV irradiation (254 nm). In addition, all clinical samples were analyzed by dot blot hybridization. For this purpose, 5  $\mu$ l of the sample was dot spotted on a GeneScreen Plus membrane (Dupont) after PCR, denatured by incubation in 0.4 M NaOH for 5 min, and hybridized with *B. pertussis*- and *B. parapertussis*-specific probes, with 20 ng of probe per 10 ml of hybridization solution, at 68°C overnight. The blots were washed at 68°C for 30 min in 1 $\times$  SSC (1 $\times$  SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-0.1% sodium dodecyl sulfate. Further washing steps were

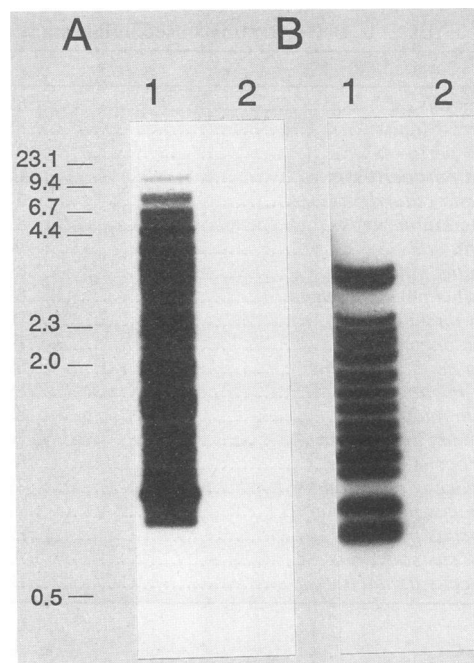


FIG. 2. Southern blot of *B. pertussis* and *B. parapertussis* chromosomal DNA probed with IS481 and IS1001. (A) Chromosomal DNA of *B. pertussis* (lane 1) and *B. parapertussis* (lane 2) digested with *Cla*I and hybridized to the IS481-specific probe. (B) Chromosomal DNA of *B. parapertussis* (lane 1) and *B. pertussis* (lane 2) digested with *Bam*HI and hybridized to the IS1001-specific probe. The sizes and positions of the lambda *Hind*III marker DNA are indicated in kilobases on the left.

carried out at room temperature according to the instruction of the hybridization kit (AMPPD; Boehringer). The membranes were exposed for variable lengths of time to X-Omat film (Kodak), generally for 1 h.

**Serology.** The preparation of antigen used in the analysis of sera has been described elsewhere (16). Details of the serological analysis of paired samples will be published elsewhere (18a). In short, a serum sample was taken both simultaneously with the nasopharyngeal swab and 2 weeks later. The occurrence of a rise of immunoglobulin G and/or immunoglobulin A antibody titers of more than fourfold was considered proof of current *B. pertussis* infection.

## RESULTS

**Sensitivity of the detection.** As DNA templates for the PCR primers, insertion sequence elements IS481 and IS1001 (18) were used. IS481 is present in about 80 copies in the chromosome of *B. pertussis* (Fig. 2, lane 1A) but has not been detected in *B. parapertussis* (10, 11). IS1001 is found in the chromosome of *B. parapertussis* in about 20 copies (Fig. 2, lane 2B) but is absent from *B. pertussis* (18). Both elements are sporadically detected in *B. bronchiseptica* strains (unpublished data). The target DNAs for PCR on these insertion sequence elements were chosen so that amplification of *B. pertussis* or *B. parapertussis* DNA would result in DNA fragments of different sizes (Fig. 1), allowing the combination of the two primer sets in a single sample. Amplification of *B. pertussis* and *B. parapertussis* with the primer sets indicated that the primers were specific and

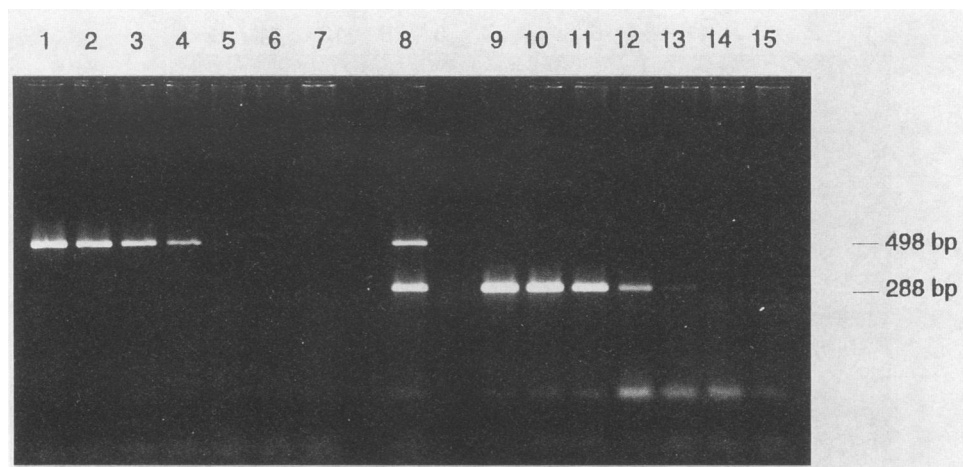


FIG. 3. Sensitivity of detection of *B. paraptentussis* and *B. pertussis* by PCR. Tenfold serial dilutions of bacteria were made in PE, and subsequently cells were lysed by heating at 100°C (method E). Lanes: 1,  $10^3$  *B. paraptentussis* cells; 2,  $10^2$  *B. paraptentussis* cells; 3,  $10$  *B. paraptentussis* cells; 4, 1 *B. paraptentussis* cell; 5, equivalent of  $10^{-1}$  *B. paraptentussis* cells; 6, equivalent of  $10^{-2}$  *B. paraptentussis* cells; 7, equivalent of  $10^{-3}$  *B. paraptentussis* cells; 8, DNA markers; 9,  $10^3$  *B. pertussis* cells; 10,  $10^2$  *B. pertussis* cells; 11,  $10$  *B. pertussis* cells; 12, 1 *B. pertussis* cell; 13, equivalent of  $10^{-1}$  *B. paraptentussis* cells; 14, equivalent of  $10^{-2}$  *B. pertussis* cells; 15, equivalent of  $10^{-3}$  *B. pertussis* cells. The sizes and positions of the DNA markers are indicated on the right.

rendered DNA fragments with the expected sizes (see below).

To investigate which method of sample preparation would render the highest sensitivity of detection, different methods (methods A through E, described in Materials and Methods) of sample preparation were investigated. After PCRs and electrophoresis were performed, the sensitivity of detection was judged by the visibility of the amplified DNA fragments on agarose gels. It was found that when cells were diluted in PE and lysed by incubation at 100°C (method E), one bacterium or less could be detected by the PCR assay (Fig. 3). In spite of the fact that the target DNA (IS1001) used to detect *B. paraptentussis* is found in about four times fewer copies than the target DNA (IS481) used for *B. pertussis*, the sensitivity of detection was approximately the same in both cases (Fig. 3). Method D, in which cells were suspended and lysed by being boiled in physiological salt, gave results identical to those obtained with method E (data not shown). In general, method C, in which cells were diluted and lysed by being boiled in H<sub>2</sub>O, was as sensitive as methods D and E (data not shown). However, method C gave less-reproducible results. Concentration or purification of DNA by ethanol precipitation or adsorption to diatoms following proteinase K treatment (respectively, methods A and B) resulted in a decrease in sensitivity of detection to approximately 100 bacteria per reaction, presumably because of loss of DNA. On the basis of these observations, we chose method E, and in all subsequent experiments, samples were prepared according to this method.

To study possible inhibitory effects on the PCRs by compounds present in clinical samples, *B. pertussis* cells were diluted in saliva and nasal washings derived from four different healthy persons. Tenfold serial dilutions of cells, ranging from  $0.2 \times 10^6$  to  $0.2 \times 10^2$  cells per ml, were made in saliva or nasal washings. Subsequently, the samples were heated for 15 min at 100°C, and 5  $\mu$ l was used for PCR. No loss of sensitivity to detect *B. pertussis* was observed compared with that for samples prepared in PE (method E). Thus, no evidence was found for inhibitory compounds in saliva or nasal washings.

#### Simultaneous detection and discrimination of *B. pertussis*

and *B. paraptentussis*. Our ultimate goal was to develop a PCR assay which would allow the simultaneous detection and discrimination of *B. pertussis* and *B. paraptentussis* in a single sample by combining the two primer sets. It is conceivable that such an assay would be less sensitive than a single assay, because the presence of one *Bordetella* species could affect the detection of the other species on account of the domination of one of the PCRs. This is especially true if the two species are present in different numbers.

Combination of the primer sets did not affect the sensitivity of detection when either *B. pertussis* or *B. paraptentussis* was present in the sample (data not shown). Essentially the same results as those shown in Fig. 3 were observed. To study the effect of the simultaneous presence of *B. pertussis* and *B. paraptentussis* in a sample on the sensitivity of detection, 10-fold serial dilutions of *B. paraptentussis* cells were added to a constant number ( $10^0$  to  $10^4$ ) of *B. pertussis* cells, and vice versa. The presence of up to  $10^4$  *B. paraptentussis* cells did not affect the sensitivity of detection of *B. pertussis* with the combined primer sets (Table 2). However, even the presence of a single *B. pertussis* cell decreased the

TABLE 2. Effect of the simultaneous presence of *B. pertussis* and *B. paraptentussis* on the sensitivity of the PCR<sup>a</sup>

Bpp cells/ reaction	Result with the following no. of Bp cells/reaction									
	10 <sup>4</sup>		10 <sup>3</sup>		10 <sup>2</sup>		10 <sup>1</sup>		10 <sup>0</sup>	
	Bpp	Bp	Bpp	Bp	Bpp	Bp	Bpp	Bp	Bpp	Bp
10 <sup>4</sup>	+	+	+	+	+	+	+	+	+	+
10 <sup>3</sup>	+	+	+	+	+	+	+	+	+	+
10 <sup>2</sup>	-	+	-	+	-	+	-	+	-	+
10 <sup>1</sup>	-	+	-	+	-	+	-	+	-	+
10 <sup>0</sup>	-	+	-	+	-	+	-	+	-	+

<sup>a</sup> Tenfold serial dilutions of *B. paraptentussis* (Bpp) cells were added to a constant number of *B. pertussis* (Bp) cells, and vice versa. Subsequently, PCR was performed according to method E, with 125 ng of each primer set. The numbers  $10^0$  to  $10^4$  refer to the numbers of cells in the PCR mixture. + and - indicate whether the 498-bp *B. paraptentussis*- or the 288-bp *B. pertussis*-specific fragments were or were not detected, respectively.

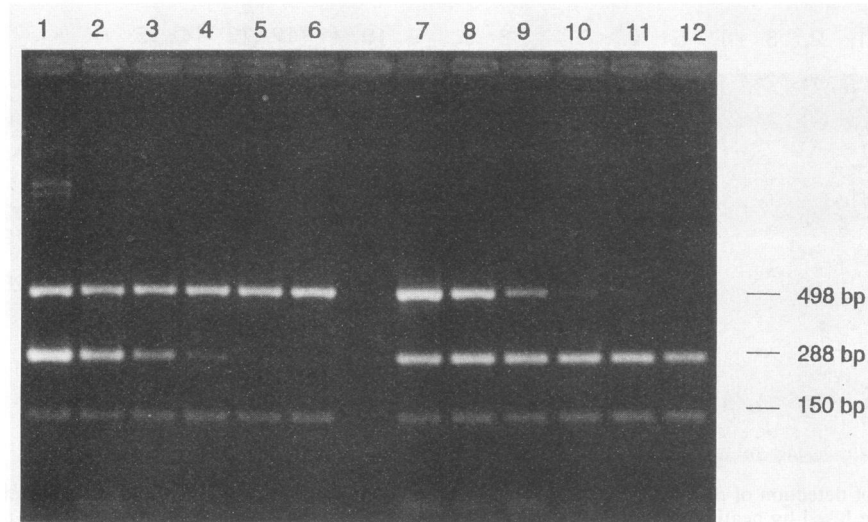


FIG. 4. Sensitivity of simultaneous detection of *B. paraptussis* and *B. pertussis* by PCR, in the presence of 50 fg of internal control DNA. Tenfold serial dilutions of bacteria were made in PE, and cells were lysed by heating at 100°C (method E). Lanes 1 to 6,  $10^2$  *B. paraptussis* cells in addition to (the equivalent of)  $10^3$ ,  $10^2$ , 10, 1,  $10^{-1}$ , and  $10^{-2}$  *B. pertussis* cells, respectively; lanes 7 to 12,  $10^2$  *B. pertussis* cells in addition to (the equivalent of)  $10^3$ ,  $10^2$ , 10, 1,  $10^{-1}$ , and  $10^{-2}$  *B. paraptussis* cells, respectively. The sizes of the amplified DNA fragments are indicated on the right.

sensitivity with which *B. paraptussis* could be detected by 10-fold (Table 2). The fact that the presence of *B. pertussis* had a more pronounced effect on the detection of *B. paraptussis* than vice versa could be due to differences in the copy number, and thus in the concentration, of the target DNA. The target DNA for the *B. pertussis* PCR is present in at least 80 copies per genome, whereas this number is 20 for *B. paraptussis*. This higher copy number could result in preferential amplification of *B. pertussis* DNA. To determine whether it was possible to compensate for the higher concentration of template, we adjusted the concentration of primer pairs to a ratio inversely reflecting the copy number of the target DNA. The template copy number effect was indeed eliminated when, respectively, 30 and 120 ng of the *B. pertussis*- and *B. paraptussis*-specific primer sets were added to the reaction mixture, corresponding to a ratio of 1:4. This ratio of primers allowed the detection of a single *B. paraptussis* cell in the presence of up to  $10^4$  *B. pertussis* cells, and vice versa (data not shown), and it was used in subsequent experiments.

**An internal control of the PCR.** Occasionally, we found that for no apparent reason, amplification of target DNA in clinical samples was blocked completely. To be able to discriminate between these false-negatives and true-negative results, we deemed it necessary to construct an internal control to monitor the performance of the PCR. The internal control consisted of a DNA fragment, contained in pRPP100, derived from *E. coli* and flanked by the primers BP1 and BPPZ (see Materials and Methods). The rationale behind the choice of this internal control was that it could not hybridize to the *B. pertussis*- and *B. paraptussis*-specific probes in the dot blot and did not require the incorporation of additional primers in the reaction mixture. Amplification of pRPP100 with the *B. pertussis*- and *B. paraptussis*-specific primers resulted in the appearance of a 150-bp DNA fragment that could clearly be distinguished from the *B. pertussis*- and *B. paraptussis*-specific amplification products.

To determine the detection limit of the internal control, 10-fold serial dilutions of plasmid pRPP100 were tested by

PCR with the 1:4 primer ratio as described above. It was found that the 150-bp amplified DNA fragment could be detected if the reaction mixture contained 1 fg or more of pRPP100 DNA (data not shown).

To investigate the influence of the internal control on the sensitivity of detection of *B. pertussis* and *B. paraptussis*, serial dilutions of *B. pertussis* cells were added to a constant number of *B. paraptussis* cells, and vice versa, as described above. In addition to *Bordetella* target DNA, the mixtures contained 50 fg of pRPP100. Addition of the internal control did not decrease the sensitivity of the PCR assay (Fig. 4). This experiment was repeated with 20 and 100 fg of pRPP100, with identical results (data not shown). Subsequent PCRs were performed in the presence of 30 fg of pRPP100 DNA.

**The specificity of the PCR.** Various commensal and potential pathogenic bacteria are normal inhabitants of the mouth and upper respiratory tract. To investigate whether DNA sequences present in these bacterial species may lead to the amplification of distinct DNA fragments with the *B. pertussis*- and *B. paraptussis*-specific primers, the strains listed in Table 1 were tested separately by means of PCR, with approximately  $10^4$  cells per reaction. Although in some cases faint bands with high molecular weights (i.e., larger than 1,000 bp) were visible, no *B. pertussis*- or *B. paraptussis*-specific amplification products could be detected after gel electrophoresis (data not shown).

**Performance of the PCR on clinical samples.** The performance of the PCR was investigated with 20 selected clinical samples from children who were suspected of having pertussis on the basis of clinical symptoms and who were subsequently diagnosed by serology and/or culture (Table 3). Since contamination of samples during processing may occur and may result in false-positive results, negative controls (one for every five clinical samples) were processed in parallel with the clinical samples. Negative controls showed only the amplified 150-bp product derived from the internal control. In all clinical samples except one (sample I), the 150-bp fragment was detected, indicating that clinical spec-

TABLE 3. Analysis of selected clinical samples with PCR<sup>a</sup>

Patient	Result by		
	PCR <sup>b</sup>	Culture	Serology
A	+	-	+
B	+	-	+
C	+	-	+
D	+	-	+
E	+	-	+
F	+	+	+
G	+	+	+
H	+	-	+
I <sup>c</sup>	-/+	+	ND
J <sup>d</sup>	+/-/-	+/-/-	ND
K-T <sup>e</sup>	-	-	-

<sup>a</sup> The samples were obtained from patients suspected of having pertussis and were prepared according to method E. ND, not determined.

<sup>b</sup> + and - indicate whether *B. pertussis*-specific amplification products were detected or not. *B. parapertussis* was not detected in this study. In all samples except sample I, the 150-bp fragment derived from the internal control was visible after PCR.

<sup>c</sup> Since the internal control was initially not detected in the sample taken from this patient, it was extracted with phenol-chloroform and tested again. After extraction, the 288-bp *B. pertussis*-specific fragment and the 150-bp fragment derived from the internal control were detected. Results of the PCR before and after phenol-chloroform extraction, respectively, are indicated.

<sup>d</sup> This patient was treated with erythromycin from day 1 on. Results of PCR and culture for samples taken on days 1, 10 and 17, respectively, are shown.

<sup>e</sup> In the samples taken from these patients, only the 150-bp fragment, derived from the internal control, was visible.

imens obtained by nasal swabs and prepared according to method E generally do not contain inhibitors of the PCR. Ten samples (K through T), obtained from patients which were classified as pertussis negative according to culture and serology, were also negative in the *B. pertussis*- and *B. parapertussis*-specific PCR. These samples were probably derived from patients infected with respiratory pathogens other than *B. pertussis* and *B. parapertussis*. In seven samples (A through H), derived from patients who were diagnosed as positive for pertussis on the basis of serology, the 288-bp *B. pertussis*-specific fragment was detected. In two of these patients, pertussis was confirmed by culture. In sample I, no amplification products were detected, suggesting that this sample contained substances that inhibited the PCR. After phenol-chloroform extraction, the 150-bp internal control fragment and the 288-bp *B. pertussis*-specific fragment were detected, indicating that this treatment sufficed to remove the inhibitors. No serological data were available from the corresponding patient, but the result of the PCR was confirmed by culture. From one patient (J), who received erythromycin treatment from day 1 on, follow-up samples were collected at days 10 and 17. The samples taken at days 1 and 10 gave positive PCR results, while the result for culture was positive only on day 1. This suggests that most bacteria were nonviable on day 10 but still detectable with PCR. On day 17, both PCR and culture gave negative results. No serological data were available from this patient.

**Confirmation by dot blot analysis.** Occasionally we observed PCR products in clinical samples which differed in size from the *B. pertussis*- and *B. parapertussis*-specific products. These fragments were possibly generated by non-specific annealing of the primers to DNA targets derived from organisms other than *B. pertussis* and *B. parapertussis*. It is conceivable that in some cases these nonspecific fragments may have the same size as *B. pertussis*- and *B.*

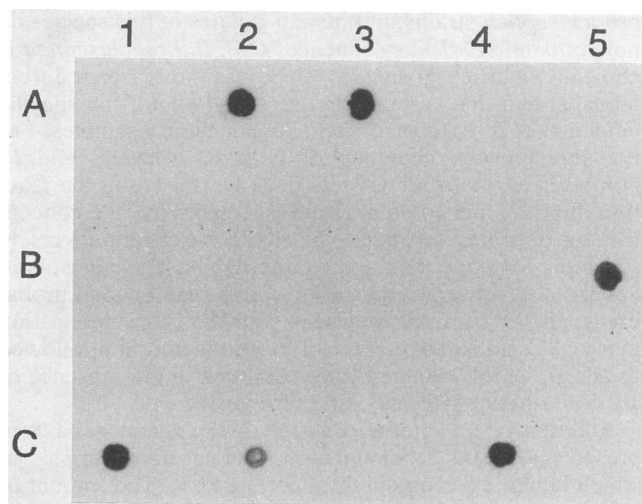


FIG. 5. Dot blot hybridization of amplified DNA products from clinical samples with a *HpaI-ClaI* fragment of IS481 as a probe. *B. parapertussis* (A1)-amplified DNA served as a negative control, and *B. pertussis* (A2)-amplified DNA served as a positive control. Spots A3, B5, C1, C2, and C4 contained clinical samples in which the 288-bp *B. pertussis*-specific amplification product was detected. Spots A4 and A5 contained clinical samples with amplification products that migrated at a position slightly different from that of the 288-bp *B. pertussis*-specific fragment. Spots B1, B2, B3, B4, and C3 contained clinical samples in which *B. pertussis*- and *B. parapertussis*-specific amplification products were not detected.

*parapertussis*-specific amplification products, resulting in a false-positive PCR. To verify whether *B. pertussis*-specific template DNA situated between the primers was amplified in clinical samples, PCR products from a number of clinical samples were dot spotted and hybridized to the *B. pertussis*-specific probe (Fig. 5). Amplified DNA products from all clinical samples, containing the 288-bp *B. pertussis* fragment, reacted with the *B. pertussis*-specific probe. In two clinical samples (i.e., A4 and A5), amplified products were observed in agarose gels that migrated at a position slightly different from that of the 288-bp fragment, and these fragments did not react with the *B. pertussis* probe. None of the samples that were PCR negative as judged by gel electrophoresis reacted with the probe (Fig. 5).

## DISCUSSION

A PCR-based detection of pathogens by amplification of specific DNA sequences theoretically allows the detection of a single bacterium. The use of repeated DNA sequences for PCR is attractive, since this enhances the sensitivity of the detection compared with that for single-copy sequences. A repeated DNA sequence specific for *B. pertussis*, designated IS481 (10, 11), has previously been used successfully for the detection of this species, and detection limits of three and six bacteria were reported (4, 5). We have added a number of significant improvements to the PCR-based diagnosis of pertussis: the simultaneous detection and discrimination of *B. pertussis* and *B. parapertussis* in a single sample, the inclusion of an internal control to monitor the PCR, and confirmation of the PCR results by means of dot blot hybridization.

The detection of *B. parapertussis* by means of PCR was based on the sequence of IS1001, which is present in 20 copies per cell. A similar sequence is also found in some *B.*

*bronchiseptica* strains, but human isolates of this species do not contain IS1001-like sequences (18). *B. bronchiseptica* is clinically of little significance, since it is rarely isolated from humans, and this permits the use of IS1001 for the specific detection of *B. parapertussis* in human clinical samples. For the simultaneous detection of both *B. pertussis* and *B. parapertussis*, two primer sets must be present in the reaction mixture, and to obtain optimal sensitivity, the concentrations of primer sets had to be adjusted to a ratio inversely reflecting the copy number of target DNA. This adjustment appeared to prevent domination of one reaction and probably levels the starting conditions after the first amplification cycles. The adjustment of the primer concentrations allowed the detection of a single *B. pertussis* cell in the presence of  $10^4$  *B. parapertussis* cells, and vice versa.

Addition of an internal control to the reaction mixture presents a means to distinguish false-negative samples in which inhibitory compounds affect the PCR. The amount of internal control added to the reaction mixture was chosen such that amplification of the 150-bp control target was highly reproducible. Discrimination of amplified DNA derived from the internal control, *B. pertussis*, or *B. parapertussis* was accomplished by differential spacing of the primers. The sensitivity of the combined PCR method was found to be very high and allowed the detection of a single cell of either pathogen.

Hybridization of amplified DNA with specific probes can be performed to substantiate the results obtained with the PCR assay. With the dot blot hybridization described here, large numbers of samples can be processed, and the procedure can be carried out in most clinical laboratories, especially if nonradioactive detection methods are used, as described in this study. In general, blotting and hybridization may be omitted since it is unlikely that nonspecific amplification products will have molecular sizes identical to those of the *B. pertussis*- and *B. parapertussis*-specific fragments. Of the many (approximately 600) samples that we have tested to date, only two gave rise to amplification products which migrated close to the *B. pertussis*-specific fragment. Dot blot analysis indicated that these products did not hybridize to IS481 DNA (Fig. 5).

The usefulness of the PCR assay was evaluated with a number of selected clinical samples from children suspected of having pertussis. The results obtained with PCR were compared with those from culture and/or serology (Table 3). Ten suspected pertussis samples were confirmed by culture or serology, and nine of these samples yielded a positive signal as determined by PCR. In the sample derived from the remaining pertussis patient, the internal control was also not detected, suggesting the presence of inhibitors. Phenol-chloroform extraction restored the amplification of the internal control and also resulted in the appearance of the *B. pertussis*-specific amplification product. These results underline the importance of including an internal control in PCR-based diagnosis. Recently, we have also detected *B. parapertussis* in clinical samples from suspected patients of having pertussis by using the PCR assay (18a). At present, a larger number of clinical samples are being collected. These samples will be used to compare the sensitivity and specificity of the PCR assay with those of culture and serology.

The PCR assay described in the present study may prove to be an improvement of the present methods for surveillance of pertussis and may provide a more accurate means to determine the efficacy of new, acellular pertussis vaccines

and their long-term effects on the incidence of *B. pertussis* and *B. parapertussis* infections.

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#### REFERENCES

1. Amano, K., K. Fukushi, and M. Watanabe. 1990. Biochemical and immunological comparison of lipopolysaccharides from *Bordetella* species. *J. Gen. Microbiol.* **136**:481-487.
2. Boom, R., C. J. A. Sol, M. M. M. Salimans, C. L. Jansen, P. E. Wertheim-van Dillen, and J. van der Noordaa. 1990. Rapid and simple method for purification of nucleic acids. *J. Clin. Microbiol.* **28**:495-503.
3. Dente, L., M. Sollazo, C. Baldari, G. Cesareni, and R. Cortese. 1985. The pEMBL family of single-stranded vectors, p. 101-107. In D. M. Glover (ed.), *DNA cloning—a practical approach*, vol. 1. IRL Press, Oxford.
4. Glare, E. M., J. C. Paton, R. R. Premier, A. J. Lawrence, and I. T. Nisbet. 1990. Analysis of a repetitive DNA sequence from *Bordetella pertussis* and its application to the diagnosis of pertussis using the polymerase chain reaction. *J. Clin. Microbiol.* **28**:1982-1987.
5. Houard, S., C. Hackel, A. Herzog, and A. Bollen. 1989. Specific identification of *Bordetella pertussis* by the polymerase chain reaction. *Res. Microbiol.* **140**:477-487.
6. Khelef, N., B. Danve, M. J. Quentin-Millet, and N. Guiso. 1993. *Bordetella pertussis* and *Bordetella parapertussis*: two immunologically distinct species. *Infect. Immun.* **61**:486-490.
7. Linneman, C. C., Jr., and E. B. Pery. 1977. *Bordetella parapertussis*: recent experience and a review of the literature. *Am. J. Dis. Child.* **131**:560-563.
8. Long, S. S., H. W. Lischner, A. Deforest, and J. L. Clark. 1990. Serologic evidence of subclinical pertussis in immunized children. *J. Pediatr. Infect. Dis.* **9**:700-704.
9. Long, S. S., C. J. Welkon, and J. L. Clark. 1990. Widespread silent transmission of pertussis in families: antibody correlates of infection and symptomatology. *J. Infect. Dis.* **161**:480-486.
10. McLafferty, M. A., D. R. Harcus, and E. L. Hewlett. 1988. Nucleotide sequence and characterization of a repetitive DNA element from the genome of *Bordetella pertussis* with characteristics of an insertion sequence. *J. Gen. Microbiol.* **134**:2297-2306.
11. McPheat, W. L., J. H. Hanson, I. Livey, and J. S. Robertson. 1989. Analysis of separate isolates of *Bordetella pertussis* repeated DNA sequences. *J. Gen. Microbiol.* **135**:1515-1520.
12. Mertsola, J. Mixed outbreak of *Bordetella pertussis* and *Bordetella parapertussis* infection in Finland. 1985. *Eur. J. Microbiol.* **4**:123-128.
13. Mertsola, J., T. Kuronen, A. Turunen, M. K. Viljanen, and O. Ruuskanen. 1984. Diagnosis of pertussis. *J. Infect.* **8**:148-156.
14. Mooi, F. R., H. G. J. van der Heide, A. R. ter Avest, K. G. Wellinder, I. Livey, B. A. M. van der Zeijst, and W. Gaastra. 1987. Characterization of fimbrial subunits from *Bordetella* species. *Microb. Pathog.* **2**:473-484.
15. Mortimer, E. A. 1990. Pertussis and its prevention: a family affair. *J. Infect. Dis.* **161**:473-479.
16. Nagel, J., S. de Graaf, and D. Schijff-Evers. 1985. Improved serodiagnosis of whooping cough by *Bordetella pertussis* by determination of IgG anti-LPF antibody levels. *Dev. Biol. Stand.* **61**:325-330.
17. Storsaeter, J., and P. Olin. 1992. Relative efficacy of two acellular pertussis vaccines during three years of passive surveillance. *Vaccine* **10**:142-144.
18. van der Zee, A., C. Agterberg, M. van Agterveld, M. Peeters, and F. R. Mooi. 1993. Characterization of IS1001, an insertion sequence element of *Bordetella parapertussis*. *J. Bacteriol.* **175**:141-147.
- 18a. van der Zee, A., C. Agterberg, M. Peeters, J. Schellekens, and F. R. Mooi. Unpublished data.