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Nested PCR Optimized for Detection of *Bordetella pertussis* in Clinical Nasopharyngeal Samples

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Several genes and sequences in *Bordetella pertussis* have been used as targets in diagnostic PCR assays. A previously developed single-step PCR assay for the detection of *B. pertussis* was based on an insertion sequence, IS*480*, that is present in about 70 to 80 copies in each genome. The diagnostic sensitivity, specificity, and reliability of this assay with aspirated and heat-treated samples from the nasopharynx of patients and their contacts was improved by the use of a nested PCR configuration. The nested PCR assay produced a 205-bp fragment with all of the 115 *B. pertussis* strains tested and was negative with all strains belonging to other *Bordetella* species ($n = 44$) as well as other bacteria commonly found in the upper respiratory tract ($n = 115$). The diagnostic value of the assay was verified by giving positive results for *B. pertussis* in all the 51 nasopharyngeal aspirates from culture-positive patients. The assay also detected 18 positive aspirates from a total of 196 culture-negative patients. A confirmatory cleavage of the 205-bp nested PCR product by *Mva*I gave in all cases two bands of 88 and 117 bp. In conclusion, this newly developed nested PCR assay was shown to be reasonably fast and uncomplicated, with an optimal sensitivity and a high degree of specificity for the diagnosis of *B. pertussis* in aspirated nasopharyngeal samples processed simply by heat treatment. The detection level in the nested PCR was about 10 bacteria per ml, or seven to eight insertion sequence copies per 10 μl of boiled sample.

The specific diagnosis of pertussis is important for several reasons, e.g., for treatment and epidemiological interventions as well as evaluation of vaccine efficiency. To date this has mainly depended on culture and serology. Culture has, however, a low sensitivity (5) and serology is not helpful in the acute phase of the disease, and the culture result for samples from, for example, vaccinated children can be difficult to interpret (7). Antigen detection by immunofluorescence and enzyme-linked immunosorbent assay techniques as well as tests for adenylate cyclase activity (2) have therefore been tried as diagnostic tests. In addition, DNA hybridization (3, 19, 22) and PCR (24) assays have been developed in recent years (4). The PCR method is sensitive and reasonably fast. Several different regions in *Bordetella pertussis* DNA, such as the repeated insertion sequence (4, 9, 11, 13, 18, 26), the adenylate cyclase toxin gene (1), the DNA upstream of the porin gene (16), the DNA in the 16S-23S rRNA spacer sequences (23), and the pertussis toxin promoter region (6, 11, 21), have been used as targets for this method.

A previous study (18) was based on a single-step PCR for the diagnosis of pertussis by detection of a 400-bp fragment of the insertion sequence IS*480* (12, 14, 15), which is about 1,050 bp long and exists at 70 to 80 copies per genome in *B. pertussis* (4, 9, 13, 26). No cross-reactions were seen with the other species within the genus *Bordetella*. However, IS*480* has been shown to hybridize to some extent to single strains of *Bordetella bronchiseptica* (4), a species not known to cause disease in humans. This hybridization probably occurs in a region outside of the region amplified in the PCR. A problem with the use of the single-step PCR technique for routine clinical diagnosis is that it sometimes fails to amplify *B. pertussis* DNA in complex clinical nasopharyngeal samples because of the presence of inhibitors (25) or other factors. In a nested PCR assay (17) DNA is first amplified with a set of outer primers. This is followed by a second amplification with so-called nested primers covering a selected sequence within the primarily amplified DNA sequence. An advantage with a nested PCR system is that it gives a significant dilution of the original material, including inhibiting substances. In addition, the use of a nested system theoretically gives a built-in confirmation of the primarily amplified region by the nested primers. In order to increase the efficiency of the single-step PCR assay (18), which had a sensitivity of 0.76 in a previous study (18), a nested pair of primers was designed with the aim of obtaining a robust, sensitive, and specific PCR assay for the more efficient diagnosis of *B. pertussis* in nasopharyngeal samples.

MATERIALS AND METHODS

Patients and sampling. Samples from consecutive children hospitalized or attending the outpatient clinic of the Department of Pediatrics of the Örebro Medical Center Hospital with clinical symptoms of pertussis ($n = 75$) were obtained from January to December 1990. In addition, samples from consecutive children and their contacts with clinical symptoms suggesting pertussis and participating in a national vaccination study ($n = 172$) were obtained from January 1992 to December 1993. For the PCR assay nasopharyngeal samples were taken by aspiration with a prefabricated kit for tracheal suction (Intermed; Nunc, Roskilde, Denmark). After suction via one of the nostrils for 3 s the catheter was withdrawn. In the samples from 1990, 5 to 10 ml of physiological saline was then aspirated to wash the catheter and the collected fluid was frozen at −70°C within 3 to 4 h. In the samples from 1992 and 1993, 1 ml of phosphate-buffered saline was used to wash the catheter. The tip of the catheter was also aseptically put in the enrichment medium of Regan and Lowe (20) for culture. The

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TABLE 1. Bacterial strains used in the nested PCR assay for *B. pertussis* DNA[a]

Bacteria	No. of isolates
Bordetella pertussis[b]	115
Bordetella parapertussis	32
Bordetella bronchiseptica	7
Bordetella avium	3
Bordetella species	2
Alcaligenes species	5
Haemophilus influenzae	22
Haemophilus parainfluenzae	4
Moraxella catarrhalis	4
Other *Moraxella* species	4
Neisseria meningitidis	7
Neisseria gonorrhoeae	4
Other *Neisseria* species	10
Streptococcus pneumoniae	12
Streptococcus groups A, B, C, and G	14
Alpha streptococci	7
Staphylococcus aureus	4
Staphylococcus epidermidis	4
Diphtheroid rods	4
Bacteroides species	2
Anaerobic streptococci	4
Fusobacterium species	2
Propionibacterium acne	2

[a] The total number of strains tested was 274.
[b] All 115 isolates had a positive PCR result. All the other bacterial strains in the table had a negative PCR result.

samples were kept at −70°C and were never thawed more than twice before being assayed.

Culture. In 1990 the nasopharyngeal samples for culture for *B. pertussis* were taken via one nostril with a dacron swab (ENT Swab) that was immediately placed in the enrichment medium before transport within 3 h to the Department of Clinical Microbiology and Immunology. The swabs were cultured on enriched medium (charcoal agar with 10% defibrinated horse blood and 40 mg of cephalexin per liter; Oxoid, Basingstoke, United Kingdom) at 36°C with high humidity, and the cultures were examined for growth of *B. pertussis*-like colonies for up to 7 days. The cultured swab was reinserted in the transport medium immediately after culture and was recultured in the same way after 3 days of incubation at 36°C. In 1992 and 1993, culture for *B. pertussis* was performed on the aspirate (swabs were not taken) in the same way by inoculating three drops (approximately 90 μl) of aspirated fluid from a Pasteur pipette. The tip of the chopped catheter was also cultured after 3 days of incubation at 36°C.

The diagnosis of *B. pertussis* was based on typical colony forms, direct microscopic observation of short gram-negative rods, oxidase and catalase positivity, a specific reaction with agglutinating anti-*B. pertussis* serum (produced at the National Bacteriological Department, Stockholm, Sweden, or purchased from Difco Laboratories), and a negative reaction by the agglutination test with anti-*B. parapertussis* serum (Difco Laboratories).

Bacterial strains. In order to examine the sensitivity and specificity of the nested PCR assay 266 clinical and 8 reference strains were used (Table 1). The clinical strains were chosen to represent the genus *Bordetella* and bacteria commonly found in the upper respiratory tract. These strains were collected between 1983 and 1991 (*Bordetella* species) and between 1976 and 1991 (other bacteria) from Örebro County, Sweden. The American Type Culture Collection (ATCC) reference strains included were *B. pertussis* ATCC 9340, ATCC 9797, and ATCC 9340, *B. parapertussis* ATCC 15311 and ATCC 15237, *B. bronchiseptica* ATCC 786 and ATCC 19395, and *B. avium* ATCC 35086.

TABLE 2. Results by nested PCR for *B. pertussis* in relation to results by nasopharyngeal culture of swabs and aspirates[a]

Nested PCR result	No. of samples with the indicated culture result:					
	Swab			Aspirate		
	Positive	Negative	Total	Positive	Negative	Total
Positive	25	7	32	26	11	37
Negative	0	43	43	0	135	135
Total	25	50	75	26	146	172

[a] Swabs were obtained in 1990, and aspirates were obtained in 1992 and 1993.

Nested PCR assay and restriction enzyme verification of amplified product. A nested PCR method was developed from the single-step PCR assay described before (18). The new primers were designed to give a 205-bp fragment (base pairs 634 to 839 on IS*480*) (12). The PCR assays were performed in a laboratory where the handling of reagents and samples was physically separate from that of amplified material. As positive and negative controls, a clinical isolate of *B. pertussis* (strain 91-12-0222; 7×10^4/ml in water) and a nasopharyngeal aspirate from a healthy individual were used, respectively. Controls were included in each amplification, and the clinical samples were assayed and interpreted without knowledge of the culture results. The nasopharyngeal samples were boiled for 30 min in capped Eppendorf tubes, and 10 μl was added to give a final PCR mixture of 30 μl containing 1× reaction buffer (1.5 mM Mg^{2+}; Perkin-Elmer Cetus), 200 μM deoxynucleoside triphosphates (Perkin-Elmer Cetus), 1 U of AmpliTaq polymerase (Perkin-Elmer Cetus), 15% glycerol, and 0.33 μM (each) primer (5′-GACTTCGTCTTCGTGGCCAT-3′ and 5′-GTA CAGCGCGCCCGATGCCT-3′; Symbicom, Umeå, Sweden). The reaction mixture was covered with mineral oil and amplified in a DNA thermal cycler (Perkin-Elmer Cetus). Each cycle consisted of temperature steps at 94, 50, and 72°C as described before (18). The first round of the nested PCR was for 20 cycles. From this reaction mixture a 1-μl sample was added to a new PCR mixture (29 μl) containing the new nested primers (5′-CGCGTGGCCTTCACCGACAT-3′ and 5′-GGGCGGT AAGGTCGGGTAAA-3′; Scandinavian Gene Synthesis AB, Köping, Sweden). This PCR was run under the same conditions described above, but for 30 cycles. The PCR product (8 μl) was assayed on a 3% agarose gel (Nusieve GTG; FMC Bioproducts) with 40 mM TAE (Tris-acetate, EDTA) buffer and ethidium bromide and the band positions were compared with those of DNA molecular weight marker V (Boehringer, Mannheim, Germany) as described before (18). To confirm the identity of the product, all culture-negative samples and a random selection of the culture-positive samples were digested with *Mva*I for 1 h at 37°C and were electrophoresed on a 3.5% agarose gel. The detection limit was investigated by amplification of boiled dilutions of *B. pertussis* 91-12-0222 in the nasopharyngeal aspirate from a healthy individual. The results were analyzed on an agarose gel as described above.

RESULTS

The nested PCR assay gave a 205-bp product with all 112 clinical isolates of *B. pertussis* in water (7×10^4 bacteria per ml), the three ATCC strains of *B. pertussis*, and a mixture of *B. pertussis*, *Haemophilus influenzae*, *Streptococcus pneumoniae*, and a *Moraxella* species (each with 10^6 bacteria per ml). All

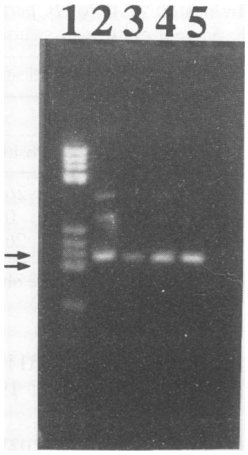

FIG. 1. Agarose gel electrophoresis after nested PCR of *B. pertussis* DNA in nasopharyngeal samples. Lane 2, PCR-positive result for swab culture-positive sample; lane 3, PCR-positive result for swab culture negative sample; lane 4, PCR-positive result for aspirate culture-positive sample; lane 5, PCR-positive result for aspirate culture-negative sample. The bands (205 bp) were compared with the 192- and 213-bp bands (marked with arrows) of the molecular mass marker in lane 1.

other *Bordetella* species (n = 44) and other respiratory tract strains (n = 115) were negative in the assay (Table 1). The nested PCR was positive with all 51 nasopharyngeal aspirates from *B. pertussis* culture-positive patients (Table 2 and Fig. 1). No strains of *B. parapertussis* or *B. bronchiseptica* were isolated from this material. A random selection of 14 of these 51 positive PCR products was subjected to *Mva*I cleavage. All of them gave two bands of the expected sizes for *B. pertussis*, i.e., 117 and 88 bp (Fig. 2). The nasopharyngeal aspirates from culture-negative patients (n = 196) were negative by nested PCR for 178 of the samples and positive for 18 of the samples

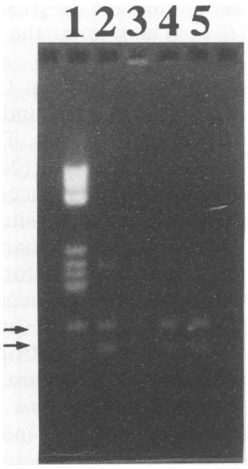

FIG. 2. Agarose gel electrophoresis after nested PCR and *Mva*I cleavage of *B. pertussis* DNA in the nasopharyngeal samples presented in Fig. 1. Lane 2, positive result for swab culture-positive sample; lane 3, positive result for swab culture-negative sample; lane 4, positive result for aspirate culture-positive sample; lane 5, positive result for aspirate culture-negative sample. The bands (117 and 88 bp) were compared with the 89- and 123-bp bands (marked with arrows) of the molecular mass marker in lane 1.

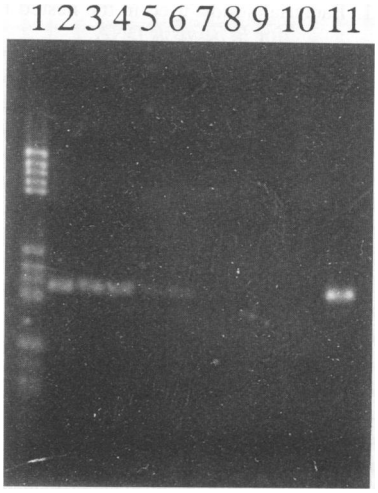

FIG. 3. Agarose gel electrophoresis after nested PCR of dilutions of heat-treated *B. pertussis* 91-12-0222 in a nasopharyngeal aspirate from a healthy individual. Lane 2, original suspension; lanes 3 to 9, a series of dilutions with 100, 50, 25, 10, 5, 1, and 0.5 bacteria per ml, respectively; lane 10, a negative control of a nasopharyngeal aspirate from a healthy individual; lane 11, positive control. The bands (205 bp) were compared with the 192- and 213-bp bands of the molecular mass marker in lane 1. A faint but clear band could be seen on the original gel in lane 6.

(Table 2; Fig. 1, lanes 3 and 5). All 18 PCR products could be verified by *Mva*I cleavage (Fig. 2, lanes 3 and 5). Altogether, the sensitivity of the nested PCR assay for the diagnosis of infection caused by *B. pertussis* in the two clinical groups was 1.00. The specificity was 0.86 when swab culture and 0.92 when aspirate culture were used as the reference methods (Table 2). The detection level for *B. pertussis* in a nasopharyngeal aspirate from a healthy individual by the nested PCR assay was determined by serial diluting boiled material to about 10 *B. pertussis* cells per ml (Fig. 3), corresponding to seven to eight copies of the target sequence per sample for the nested PCR.

DISCUSSION

The nested PCR assay for the diagnosis of *B. pertussis* in nasopharyngeal aspirates showed an optimal sensitivity in clinical samples. The targeted region was also documented in all 115 strains of *B. pertussis* examined. With culture as the reference method for diagnosis, some samples were identified to be culture negative and nested PCR positive. This finding was expected because of the well-known fact that culture is falsely negative in a proportion of cases (5). This is mainly dependent on when in the course of the disease the samples were obtained from the patients, the sampling technique, means and times of transport (10), culture system and medium, and age and whether the patient had been vaccinated or treated with antibiotics. Samples for culture were obtained from our patients earlier in the course of disease during the 1992 to 1993 period (data not shown), which may be one explanation of why only 8% were culture negative and nested PCR positive during this period whereas 14% from the previous period were culture negative and nested PCR positive. Another factor could be that culture of nasopharyngeal aspirate is more efficient than culture of swab, as documented by Hallander et al. (7).

For further exploration of the specificity of the nested PCR, a "gold standard" for the diagnosis must be created by using not only culture but also serology and preferably also PCR assays covering other parts of the genome. A study that will use this approach is planned. In clinical samples the presence of inhibitors of the PCR assay is always a potential problem, and different approaches, depending on the clinical material, to overcoming these difficulties must be evaluated. For the frozen nasopharyngeal samples, boiling in combination with the dilution which takes place during the nested PCR seems to overcome this problem in the diagnosis of pertussis by PCR. It would, however, be helpful to have an indicator of the performance of the PCR assay and a possibility of obtaining a quantitative measure of the original amount of *B. pertussis* DNA. By adding defined amounts of a labelled piece of IS*480* to every clinical sample, a competitive assay which can give information about the inhibitory activities in the sample as well as a quantitative numeric measure can be designed; this approach is being evaluated. The role of freezing compared with that of examination of fresh samples should also be evaluated. In some situations the diagnosis of *B. parapertussis* is important, although the impact on human morbidity is much less, and epidemics hardly exist. In vaccination studies a diagnostic PCR assay for *B. pertussis* ought to be included, while diagnosis of *B. bronchiseptica* infection could be considered less important unless it creates problems with false-positive results in tests for the other *Bordetella* species (8).

Recommendations for the use of PCR for the diagnosis of *B. pertussis* have been proposed (16), and the assay described here complies with most of those guidelines. The assay works for both aspirate and swab samples, and no extraction step is necessary, thus making the test simple and reliable. The assay was designed to detect *B. pertussis* and no other *Bordetella* species. The sensitivity was validated by positive results for all *B. pertussis* culture-positive samples and all *B. pertussis* strains obtained from different periods between 1983 and 1991. Strains isolated from other geographic areas remain to be examined. A false-positive control was maintained by physical separation of the reagent mixture preparation, sample storage and pretreatment, amplification, and gel electrophoresis with subsequent photography. The results for the coded samples with positive results were confirmed with typical restriction enzyme fragments on a gel. A problem with the nested PCR assay compared with a single-step PCR assay as a routine diagnostic method can be the increased risk of carryover of amplified DNA and the fact that this cannot be combated with the uracil-*N*-glycosylase carryover prevention system. Therefore, we recommend that only laboratories with thorough experience with PCR use the nested PCR technique for routine diagnostic purposes.

In conclusion the set of primers and the nested PCR approach used in the present study seem to be powerful tools and a complement to culture in the diagnosis of pertussis in the acute stage of disease. This improvement in the diagnosis of pertussis would substantially strengthen the assessment of clinical vaccine trials.

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