Immunomagnetic Separation and Solid-Phase Detection of *Bordetella pertussis*

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In the present study, novel solid-phase methods were used for both sample preparation and PCR detection of *Bordetella pertussis*. The sample preparation was performed by immunomagnetic separation with paramagnetic beads coated with polyclonal antibodies directed toward the surface antigens of the bacteria. The precoated immunobeads were directly used on nasopharyngeal aspirates to capture the bacteria on the solid support and were subsequently transferred to the PCR tube with no further manipulations. The region encompassing the pertussis toxin promoter was analyzed to allow direct discrimination between the three major *Bordetella* species (*B. pertussis*, *B. parapertussis*, and *B. bronchiseptica*). The resulting amplicons were captured on a second magnetic solid phase, allowing detection and restriction analysis of the target sequence. A colorimetric detection system based on a DNA binding fusion protein enabled the use of standardized enzyme-linked immunosorbent format tests both for the detection of *Bordetella* spp. and for species evaluation. When the optimized system was evaluated on 55 clinical aspirate samples, 21 of 22 (95%) culture-positive samples were positive by the system that we developed. In addition, two samples were positive by the PCR-based assay, while the culture assay was negative. The implications of these results are discussed.

The bacterium *Bordetella pertussis* is the causative agent of whooping cough, which is an infectious disease that occurs worldwide with a high incidence among young, unvaccinated infants and occasional mortality. Related species are *B. parapertussis*, which causes a milder respiratory infection, and *B. bronchiseptica*, which primarily infects animals. Traditionally, the laboratory diagnosis of pertussis infection is performed by culture, and this is sometimes combined with serology (11, 26). Culture, whose use is possible only in the initial phase of the infection, is highly specific when the colonies are verified by agglutination and biochemical tests. However, the sensitivity is poor and decreases with the duration of time after onset, and under optimal conditions sensitivity is often only 50% compared with that of serology (12, 26). The sensitivity is further decreased by prolonged sample transport, vaccination, and antibiotic treatment of the patient. In addition, culture takes 3 to 7 days to complete (26). Serology is considered highly sensitive (26), but there are two major drawbacks; it cannot be used in the acute phase of the disease, and it can be difficult to distinguish between vaccine effects and a pertussis infection. Moreover, the method is considered less reliable in infants. Thus, there is a need for more rapid diagnostic methods with high degrees of specificity and sensitivity, preferably for use early in infection.

Several DNA amplification-based methods for the detection of *Bordetella* spp. have been developed; these methods amplify insertion sequences (1, 7, 13, 16, 21, 33, 37), the adenylate cyclase gene (6), or the porin gene (20). Amplification of insertion sequences is usually specific for *B. pertussis*, but it sometimes also amplifies *B. bronchiseptica* DNA, and amplification of the adenylate cyclase gene cannot distinguish between the three *Bordetella* species, while use of the porin gene can differentiate *B. pertussis* from *B. parapertussis* or *B. bron-*

chiseptica. The pertussis toxin promoter region has also been used in *B. pertussis*-specific PCR-based assays (2, 8, 13, 16, 30) or to differentiate between *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* (27, 28). The amplification of pathogen-derived DNA in clinical specimens often requires initial sample preparation both to concentrate the sample in a small volume suitable for PCR and to remove polymerase inhibitors present in crude samples. Immunomagnetic separation (IMS) has been shown to be effective in recovering pathogens from urine, feces, blood, and food (14, 15, 19, 25, 31, 32).

In the study described here we investigated the possibility of using IMS for the isolation of *Bordetella* spp. from nasopharyngeal aspirates prior to PCR amplification. The diagnostic procedure includes a colorimetric detection system, which avoids the need for electrophoresis. The integrated concept (Fig. 1) consists of (i) IMS, (ii) PCR, and (iii) detection of immobilized amplified nucleic acid (DIANA) (34) in an enzyme-linked immunosorbent assay (ELISA) format together with (iv) typing of bacteria by restriction enzyme analysis (27). In the present study, only *B. pertussis* and *B. parapertussis* were detected, since the detection of *B. bronchiseptica* is of minor importance for the diagnosis of disease in humans (35). Here we show that the integrated system can be used to capture and detect *Bordetella* bacteria in clinical samples. The amplification of single *Bordetella* genomes in a homogeneous lysate is demonstrated, as is the isolation of bacteria by IMS from bacterial cultures. Fifty-five nasopharyngeal aspirates were investigated by the described approach (see Table 1).

MATERIALS AND METHODS

Patient material. Fifty-five frozen nasopharyngeal aspirates from patients participating in a phase III pertussis vaccine efficacy trial (10) and with suspected pertussis infection were obtained from The Swedish Institute for Infectious Disease Control. Twenty-two of them were positive by culture (Table 1). The numbers of colonies on the plates were estimated in four orders of magnitude: 1 to 10, 10 to 100, >100, and confluent growth. A negative aspirate sample was divided and spiked with known amounts of *B. pertussis* and *B. parapertussis* bacteria to analyze the efficiency of capture by IMS in more viscous samples compared with that in pure bacterial cultures.

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Bacterial strains and crude lysates. The sensitivity of the PCR was determined by using bacterial strains (*B. pertussis* patient isolate 324 and *B. paraptentussis* ATCC 15237) serially diluted (1:10) in phosphate-buffered saline (PBS) buffer (10 mM phosphate buffer [pH 7.5], 150 mM NaCl), and the bacteria were quantified by determining the viable counts. These pure cultures were also used for optimization of the IMS procedure and as a positive PCR control. The template used during optimization of PCR conditions was a serially diluted *B. pertussis* ATCC 9797 lysate (cultures and lysate were also obtained from The Swedish Institute for Infectious Disease Control), which was quantified as described above. This also gave an estimation of the detection limit (number of CFU) of the PCR step.

Oligonucleotide synthesis. Two sets of PCR oligonucleotides (outer oligonucleotides, STMA10-STMA11; inner oligonucleotides, STMA17-STMA18 [see Fig. 2]) were synthesized by phosphoramidite chemistry on an automated DNA synthesis machine (Gene Assembler Plus; Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's manual. Primer STMA17 was synthesized with a biotin amide (Biodite; Pharmacia Biotech) at its 5' end. All primers were purified by fast-performance liquid chromatography on a 5/5 pepRPC column (Pharmacia Biotech). The four primers are complementary to sequences in the promoter region of the pertussis toxin operon, which contains nucleotide variations in *B. paraptentussis* and *B. bronchiseptica*, resulting in different recognition sites for restriction enzymes in the three species (22).

IMS. Two polyclonal serum samples, rabbit anti-*B. pertussis* (Difco Laboratories, Detroit, Mich.) and rabbit anti-*B. paraptentussis* (Difco Laboratories), were separately mixed with precoated magnetic beads (Dynabeads M-280 sheep anti-rabbit immunoglobulin G; Dynal AS, Oslo, Norway) and were incubated in PBS with gentle rotation for 30 min at room temperature. The amounts of precoated beads and polyclonal sera were 100 µg (10 µl) and 50 µl of a 1:100 dilution, respectively, per sample for each serum sample. The excess of unbound antibody was removed by washing the bead-antibody complexes with PBS. The previously washed bead-antibody complexes were then mixed and incubated with the bacterial culture in PBS in a total volume of 100 µl. When IMS was performed on nasopharyngeal aspirates, the volume was larger (up to 800 µl). The beads with the immunocaptured bacteria were washed twice with PBS and were resuspended in 10 µl of 10 mM Tris-HCl (pH 8.3). Occasionally, the resuspended beads were frozen before analysis by PCR.

In vitro amplification. A 300-bp fragment of the pertussis toxin promoter region was amplified with two sets of oligonucleotide primers in a nested procedure (see Fig. 2). The PCR mixture in both the inner and the outer amplifications consisted of 10 mM Tris-HCl (pH 8.3), 3 mM MgCl₂, 50 mM KCl, 0.1% Tween 20, 0.2 mM (each) deoxynucleoside triphosphates, 0.1 µM (each) primer, and 1 U of AmpliTaq DNA polymerase (Perkin-Elmer, Norwalk, Conn.) in total volumes of 50 µl (outer) and 75 µl (inner). The mixture was covered with two drops of light white mineral oil (Sigma Chemical Co., St. Louis, Mo.) as a barrier to prevent contamination, and 10 µl of resuspended beads was added through the layer of mineral oil. The PCR was performed with a PE9600 instrument (Perkin-Elmer). The first amplification step was run with the outer primer pair (STMA10-STMA11). The cycle conditions for the outer amplification were performed at 94°C for 5 min; this was followed by 35 cycles of 96°C for 30 s, 64°C for 30 s, and 72°C for 1 min, with a final extension at 72°C for 10 min. A total of 5 µl of the outer amplification product was used as template in the subsequent inner amplification step with the inner primer pair (STMA17-STMA18), generating the specific 300-bp inner fragment. The cycle conditions used were the same as those used during the outer amplification, except that an annealing temperature of 68°C was used. Water and *B. pertussis* lysate were used as negative and positive PCR controls, respectively. The amplified product (5 µl) was analyzed by agarose gel electrophoresis and by the DIANA system (15 µl). To avoid cross-contamination, three separate rooms were used for mixing the reagents, the addition of template, and post-PCR work. Separate pipettes were used, together with aerosol-resistant tips (Molecular Bio-Products Inc., San Diego, Calif.). Two types of negative controls were used: one was processed in parallel with the samples to check cross-contamination during immunomagnetic sample preparation and one was processed to check the PCR solutions. Amplification of clinical samples was performed blinded, without knowledge of the culture results.

DIANA. The solid phase used for analysis in the DIANA consisted of magnetic beads with covalently coupled streptavidin (Dynabeads M-280 Streptavidin; Dynal AS). The magnetic beads (30 µl; 300 µg) were mixed with 15 µl of the PCR mixture and 50 µl of LacI-β-galactosidase fusion protein (Dynal AS) in a microtiter plate (Sero-Wel; Bibby Sterilin Ltd., Stone, United Kingdom), and the mixture was incubated for 15 min at room temperature. By applying a microtiter plate magnet (MPC 96; Dynal AS), the bead-DNA-fusion protein complexes were separated and washed four times with 150 µl of DIANA buffer (0.1 M Tris-HCl [pH 7.5], 0.15 M NaCl, 0.1% Tween 20, 1 mM MgCl₂, 10 mM β-mercaptoethanol). After the last washing step, the beads were resuspended in 50 µl of DIANA buffer, and 50 µl of substrate solution (*o*-nitrophenyl-β-D-galactoside [ONPG]; 1.25 mg/ml) was added. After 5 min, 90 µl of the reaction mixture was transferred to a well containing 100 µl of stop solution (1 M Na₂CO₃ [pH 12]) and the change in the *A*₄₀₅ at room temperature was measured in an ELISA plate reader (EAR 340 AT; SLT Instruments, Salzburg, Austria).

Colorimetric restriction enzyme analysis. Samples positive by DIANA (or by agarose gel electrophoresis) can be treated with restriction enzymes to identify

FIG. 1. Principle of the IMS-PCR-DIANA method. βgal, β-galactosidase.

individual species (27). This was performed by dividing into aliquots 45 µl of amplified product immobilized onto the streptavidin-coated magnetic beads and placing the aliquots into three vials for treatment with *TaqI* (Gibco, Life Technologies), which cleaves only the amplified sequences from *B. pertussis* and *B. bronchiseptica*; with *BglI* (Pharmacia), which cleaves only those from *B. paraptentussis*; and with *HaeII* (Boehringer, Mannheim, Germany), which cleaves *B. paraptentussis* and *B. bronchiseptica* amplicons (see Fig. 2). The DNA immobilized onto the beads was restricted according to the manufacturer's recommendations. The beads were then mixed with the LacI-β-galactosidase fusion protein, and the DIANA analysis was continued as described above.

Cloning and expression of a LacI-β-galactosidase fusion protein. In the initial experiments, a previously reported LacI-β-galactosidase fusion protein (34) was used. The fusion protein was based on a genomic fusion between the *Escherichia coli* *lacI* and *lacZ* genes in strain XA90/Δ14 (5). To improve protein production, a plasmid construct encoding recombinant LacI-β-galactosidase was created.

The gene fusion was assembled between the *lacI* gene encoding the DNA-binding *lac* repressor molecule and the *lacZ* gene encoding the enzyme β-galactosidase. *E. coli* RR1ΔM15 (29) and LG90 (9) were used as bacterial hosts, and the plasmid vectors used were pRIT28 (18), pRIT34 (23), and pSKS106 (4). First, the *lacI* gene was isolated from pRIT34 by PCR cloning with the oligonucleotides STMA4 (5'-GCT AAG CTT GAA ACC AGT AAC GTT ATA CGA TG-3') and LUJO (5'-ATT CCC GGG GAT CCT CTG CCC GCT TTC CAG-3'), omitting the stop codon of *lacI*. The *lacI* gene was cloned into pRIT28, and its sequence was verified by solid-phase sequencing (17). The gene was then released with *HindIII* and *BamHI* and was inserted into similarly digested pSKS106, in frame with its *lacZ* gene. The resulting construction, pRIT35, encodes the *lacI-lacZ* fusion gene of 4,185 bp.

E. coli LG90 (*lac* mutant) harboring plasmid pRIT35 was grown in baffled Erlenmeyer flasks containing tryptic soy broth (30 g/liter; Difco) to which yeast extract (7 g/liter; Difco) and ampicillin (100 µg/ml) were added. At an optical density at 600 nm of 1, the culture was divided into two parallel batches; one was induced with 1 mM *n*-isopropyl-β-D-thiogalactopyranoside (IPTG) and the other was incubated without IPTG. The cells were harvested 2 h after induction. After washing of the cell pellets in TS buffer (0.1 M Tris-HCl [pH 7.5], 0.15 M NaCl), the cells were disrupted by sonication in TST buffer (25 mM Tris-HCl [pH 8.0], 0.2 M NaCl, 0.05% Tween 20, 1 mM EDTA) containing 10 mM β-mercaptoethanol. A comparison of the plasmid-encoded LacI-β-galactosidase and the previously used protein produced by the mutant strain XA90/Δ14 was performed.

STMA 10 5'- CTG CTC AAC CGC CAC ATC AAC GA -3'

STMA 11 5'- CCA GCC ACG TCA GCC AGC CTG TT -3'

STMA 17 5'- biotin- CAG CCC TCC AAC GCG CCA TCC C -3'

STMA 18 5'- AAT TGT TAT CCG CTC ACA ATT GCC CGA GTG CAA CGC AT -3'

FIG. 2. Locations of the primers in the pertussis toxin promoter region. Nucleotides are numbered as described by Loch and Keith (22). The amplified sequences in the outer and the inner PCRs are 499 and 300 bp, respectively. Primer nucleotides in italics correspond to the *lac* operator sequence. The different restriction enzyme sites for the three species are marked by arrows.

The β -galactosidase activity was determined in the supernatant after sonication by a colorimetric procedure with ONPG as the substrate. In addition, the DNA binding property of the fusion protein was tested by incubating the supernatants with streptavidin-coated magnetic beads with immobilized DNA containing the *lac* operator sequence, and after washing away unbound proteins, ONPG was added and the change in the A_{405} was measured.

RESULTS

Sample preparation and PCR analysis. The approach used for the detection of *Bordetella* bacteria in nasopharyngeal aspirates (outlined in Fig. 1) was based on the enrichment and purification of bacteria through IMS (14, 15, 19, 25, 31, 32). Two polyclonal rabbit sera directed against *B. pertussis* and *B. parapertussis* were used as primary antibodies. The antibodies were bound to a secondary anti-rabbit antibody, which was covalently coupled to the magnetic bead. The immunocaptured bacteria were subsequently used, without further purification, in a nested PCR. The outer primers (primers STMA10 and STMA11) were completely complementary to sequences found in the promoter region of the pertussis toxin operon, while one of the inner primers (primer STMA17) was labelled with biotin, and the other inner primer (primer STMA18) contained the *lac* operator as a handle sequence (Fig. 2). Following PCR amplification, simultaneous binding of the PCR product onto streptavidin-coated magnetic beads and binding of the recombinant *lac* repressor- β -galactosidase fusion protein was performed. Positive samples, with the *lac* operator sequence incorporated by PCR, were colorimetrically detected after the addition of the chromogenic β -galactosidase substrate ONPG (see Fig. 5). Furthermore, this assay was com-

bined with species identification on the basis of restriction enzyme analysis of PCR products immobilized onto the solid support, allowing a colorimetric determination of *Bordetella* species in a given sample (Fig. 3B).

Performance sensitivity and specificity of the PCR step. The nested PCR primers were designed to be complementary to sequences in the promoter region of the pertussis toxin gene (Fig. 2). This gene has a high degree of homology between the different *Bordetella* species analyzed and has previously been successfully used for the detection of *Bordetella* species (2, 8, 13, 16, 27, 28, 30). The specificities of the PCR primers were tested by using lysates from the three *Bordetella* species (Fig. 3A, lanes 1 to 3) and three frequently occurring non-*Bordetella* species in nasopharyngeal aspirates: *Moraxella catarrhalis*, *Haemophilus influenzae*, and *Streptococcus pneumoniae* (Fig. 3A, lanes 4 to 6, respectively). Figure 3A shows a distinct fragment of 300 bp for the *Bordetella* species, with no amplification for the other strains. This is in agreement with the results of Reizenstein et al. (27, 28), who have tested several more species by also amplifying a part of the pertussis toxin promoter.

The detection limit of the nested PCR was analyzed with a lysed *B. pertussis* culture. The culture was quantified by viable counting. A limiting dilution experiment was performed by serial dilutions of the lysate, enabling an estimate of the number of targets (3). The results of the corresponding gel electrophoresis analysis after outer and inner amplification are shown in Fig. 4. Triplicate samples of a *B. pertussis* lysate were diluted 10-fold. After outer amplification, the intensity of the fragment decreased with increasing dilution factors. A weak fragment could still be seen at the dilution corresponding to 6

FIG. 3. (A) Specificities of the PCR primers. The specificities of the PCR primers was tested on lysates from three *Bordetella* species and three common contaminating species present in nasopharyngeal aspirate samples. Lanes 1 to 3, amplified products of *B. pertussis*, *B. paraptussis*, and *B. bronchiseptica*, respectively; there was no amplification of *M. catarrhalis*, *H. influenzae*, or *S. pneumoniae* (lanes 4 to 6 respectively). Lanes 7 and 8, negative PCR controls. Bacteriophage λ DNA restricted with *PstI* was used as a marker (lane M). (B) Colorimetric typing of *Bordetella* species by DIANA combined with restriction enzyme analysis. The amplified fragments of the three species were immobilized onto streptavidin-coated beads, treated with restriction enzymes, and analyzed colorimetrically to identify species (see text for details).

CFU. After inner amplification the last dilution step amplified corresponded to 0.6 CFU. Note that the nested amplification results in similar intensities for all dilution steps, and therefore, the endpoint can easily be determined. The longer fragments generated by more concentrated bacterial lysates correspond to the outer PCR product. Importantly, to achieve good reproducibility in the end dilution experiments, the bacteria had to be lysed prior to dilution. Results from similar PCR end dilutions directly on bacterial suspensions showed both intra- and interassay variations and could not be correlated with the results from experiments with lysed cells. Gaps also appeared in the dilution series for amounts of <100 CFU (data not shown). We interpret this low level of reproducibility as an artifact of the capsule covering the bacteria, causing them to aggregate.

Isolation of bacteria by IMS. Capture of the bacteria by IMS was investigated by PCR, despite the difficulties in making representative end dilutions of bacterial cultures for the determination of the absolute concentration (see above). We chose to test the IMS step by coating polyclonal rabbit anti-*B. pertussis* and anti-*B. paraptussis* antisera onto sheep anti-rabbit immunoglobulin G magnetic beads and incubating the immunobeads with different amounts of *B. pertussis* and *B. paraptussis* in an end dilution scheme. The magnetic beads captured intact fresh or frozen bacteria on the bead surface and enabled the efficient removal of potent PCR inhibitors by magnetic separation. A precise determination was not possible, but approximately 20 CFU could be detected after IMS and PCR (data not shown). However, the subsequent clinical study, which did not involve serial dilutions, may indicate that the system enables few viable bacteria to be detected (see below). In addition, a negative nasopharyngeal aspirate was divided and spiked with known amounts of both *B. pertussis* and *B. paraptussis* organisms in cultures to estimate the efficiency of the IMS in more crude and viscous samples. The sensitivity in the spiked aspirate was the same as that in the pure cultures tested in parallel (data not shown).

Species identification. Amplification of the pertussis toxin promoter region makes it possible to distinguish between the different *Bordetella* species by restriction enzyme analysis of the amplified sequences (27), since the nucleotide variations found in *B. paraptussis* and *B. bronchiseptica* result in differ-

FIG. 4. Detection limit of the nested PCR, evaluated on a bacterial lysate. Lanes 1 to 3, 600 CFU of bacteria; lanes 4 to 6, 60 CFU; lanes 7 to 9, 6 CFU; lanes 10 to 12, 0.6 CFU; lanes 13 to 15, 0.06 CFU; lanes 16 and 17, negative PCR controls.

TABLE 1. Summary of results for the 24 samples which were positive by the PCR-based assay and/or the culture assay

Group	Specimen	Culture result (CFU)	IMS-PCR-DIANA result
A	47	<i>B. pertussis</i> (1–10)	<i>B. pertussis</i>
	11, 22, 29, 40, 43, 50 ^a	<i>B. pertussis</i> (10–100)	<i>B. pertussis</i>
	9, 10, 27	<i>B. pertussis</i> (>100)	<i>B. pertussis</i>
	6, 7, 8, 30 ^a , 31, 42, 45, 49	<i>B. pertussis</i> (confluent growth)	<i>B. pertussis</i>
	17, 19, 20	<i>B. pertussis</i> (not determined)	<i>B. pertussis</i>
B	39, 54	Negative	<i>B. pertussis</i>
C	18	<i>B. pertussis</i> (1–10)	Negative

^a Samples 30 and 50 were first negative by PCR but were positive when they were retested after dilution of the samples with PBS and mixed on a Vortex mixer to achieve more homogeneous samples.

ent recognition sites for restriction enzymes for the three species (22). To avoid analysis by gel electrophoresis, we adopted a colorimetric procedure allowing for detection (DIANA) and analysis of species. The labelled inner PCR amplicons were detected with the detection reagent LacI- β -galactosidase and a chromogenic substrate. Solid-phase identification of positive samples was performed by restriction cleavage (Fig. 2) of additional immobilized PCR products and then the addition of the detection reagent (LacI- β -galactosidase). The restricted fragments resulted in little or no signal, since the *lac* operator was removed by magnetic separation, and the intact fragments resulted in a strong colorimetric response (Fig. 3B). When the three reference *Bordetella* species were treated, the results showed that the different enzymes can be used to cleave PCR products on a solid support.

Clinical samples. Fifty-five frozen nasopharyngeal aspirates from patients with suspected pertussis infection already tested by culture were analyzed by the IMS-PCR-DIANA approach in a blinded fashion. Thirty-one of the samples were negative by both culture and the IMS-PCR-DIANA method that we evaluated. Table 1 summarizes the results for 24 samples, placed into three groups, which were positive by at least one analysis technique. Among 22 samples from which *B. pertussis* was isolated, 21 (group A) were positive by IMS-PCR-DIANA (95%). The growth estimates showed that this group contained samples with both few and many viable bacteria. Two samples (group B) were negative by culture, but they were shown to be positive when they were analyzed by the PCR-based assay. The two patients had durations of paroxysmal cough of 29 and 55 days, respectively, which should be compared with the minimal criterion of ≥ 21 days for pertussis, which was used within the trial, in accordance with a consensus meeting of the World Health Organization (10, 36). Unfortunately, serology results were missing for both patients, but one of the patients had a serology-positive sibling (data not shown). The third group (group C) consisted of one culture-positive sample which was negative by PCR. The patient who provided this sample was also positive for *B. pertussis* by serology but had no paroxysmal cough. The sample contained few bacteria, corresponding to 3 CFU. None of the patients in groups B and C had been treated with antibiotics. The colorimetric signal distribution among the samples tested showed high values for the 23 PCR-positive samples, with an average A_{405} of 0.654 (standard deviation = 0.034), while the signals for the 32 negative samples were close to background, with an average A_{405} of 0.007 (standard deviation = 0.006), making it easy to determine a positive sample (Fig. 5). Restriction enzyme typing of amplicons showed that all samples from patients in groups A and B were of *B. pertussis* in origin, and no mixed infections were observed. This was in accordance with the culture results.

DISCUSSION

Whooping cough is a highly contagious disease caused by *B. pertussis*. The diagnosis of *B. pertussis* by culture is a highly specific method, but it has a low degree of sensitivity (26), and the bacterium is fastidious, but culture is the “gold standard” in many laboratories. However, in the future the gold standard may be a DNA-based system. PCR detection has been proven to be a very promising tool for *Bordetella* detection, but it has some limitations such as robustness and the fact that it cannot distinguish viable from nonviable bacteria. Also, the sample preparation method is critical for the diagnostic sensitivity of the PCR, the two main factors being the yield of bacterial DNA from the sample and the occurrence of *Taq* DNA polymerase-inhibiting factors in clinical samples. To address these issues, we have developed a sample preparation system that

FIG. 5. Distribution of the absorbance values. The average A_{405} values for the respective groups (a total of 55 clinical samples) are shown. The maximum and minimum values are also indicated.

enables us to capture bacteria in crude samples on a magnetic support by antibody-antigen binding. The paramagnetic support allows for the rapid handling and the complete removal of inhibitors by magnetic separation. Importantly, the handling of the specimen is also simplified so that the risk of false-positive results because of laboratory contamination is lowered. Another important advantage is that intact bacterial cells can be used as the sample, since the beads with the immunocomplex are transferred directly to the PCR tube without any phenol extractions, which are generally applied to remove inhibitory substances (24, 33). The cells are simply lysed by an extended (5-min) initial heat denaturation cycle during the PCR program.

The nested primer approach has previously been used for the detection of *B. pertussis*, showing an increased robustness for complex nasopharyngeal samples (1, 21, 28). Here, we show an improved robustness by the signal distribution and the high signal-to-noise ratio among the positive samples (Fig. 5). Furthermore, the robustness of the assay is also indicated by the strong intensity of the PCR fragments in the last steps of the end dilution series (Fig. 4). Several alternatives that allow for the simultaneous typing of the different *Bordetella* species on the basis of species-specific primer sets or shared primers have been published (20, 37). In the present study we chose the pertussis toxin promoter, which enables one and the same primer set to be used without bias for the identification of the three investigated species (27). Note that sensitivity is not a major issue with this nonrepeated gene, since we detected single targets by the nested approach (Fig. 4). The attractive feature with the chosen target gene is that a non-gel-based, colorimetric system can be used to detect and distinguish the species involved (Fig. 3B). Restriction enzyme typing was performed on the DNA immobilized onto the bead surface, with subsequent evaluation performed by using the reporter fusion protein LacI- β -galactosidase. This also constitutes an extra verification step. The nature of the colorimetric assay also allows for semiquantitative analysis of samples containing more than one species. Thus, both sample preparation and detection were based on solid supports, which opens the possibility of automating the handling and analysis procedures and thereby increasing the robustness and throughput for the diagnosis of *Bordetella* species.

The present approach was evaluated with 55 clinical nasopharyngeal aspirates, and the results were compared with those obtained by culture. As shown in Table 1, a good correlation was shown for 21 samples containing different amounts of bacteria. Two more positive samples were found by PCR, while one sample with only 3 CFU on the plate was missed. This unexpectedly missed sample stresses the fact that homogeneous samples are difficult to achieve, making these comparisons somewhat difficult. Possible means of reducing viscosity could be the use of Sputolysine or similar compounds in parallel with immunomagnetic capture. Also, in order to further control the IMS step, the polyclonal antibodies, which were commercial antisera in the present study, should be exchanged for characterized monoclonal antibodies, which can be directly coupled to a solid support.

In conclusion, we have developed a reliable sample preparation step for PCR analysis based on intact (fresh or frozen) *Bordetella* bacteria. Detection and typing was performed in an ELISA-like format, enabling automated methods to be developed. The present system allows for the rapid and specific diagnosis of *Bordetella* infections and increases the possibility of the more rapid initiation of antibiotic treatment.

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