

Identification of *Bordetella pertussis* in a Critically Ill Human Immunodeficiency Virus-Infected Patient by Direct Genotypical Analysis of Gram-Stained Material and Discrimination from *B. holmesii* by Using a Unique *recA* Gene Restriction Enzyme Site

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***Bordetella pertussis* was diagnosed in a human immunodeficiency virus-infected patient by a newly developed method in which bacterial DNA is amplified directly from sputum Gram-stained slides. The validation of the method is described along with an additional new PCR-based assay that can distinguish between *B. pertussis* and *Bordetella holmesii*.**

Identification of bacteria from clinical specimens is largely done via phenotypical characterization after in vitro culture and microscopy. Not uncommonly, however, direct Gram-stained preparations reveal many organisms, yet cultures fail to demonstrate a pathogen. This was illustrated in a recent clinical case, in which a 48-year-old man with AIDS was admitted with pulmonary coccidioidomycosis. Despite antifungal therapy, his status failed to improve. Sputum samples revealed many gram-negative bacilli on direct microscopy but no pathogens on culture. Furthermore, several blood cultures grew fastidious gram-negative rods, which could not be speciated by conventional methods.

We decided to attempt identification of the two isolates by genotypical methods using universal oligonucleotide primers aimed at the small subunit rRNA (16S rRNA) gene. From the respiratory specimens, however, only Gram-stained slides were available. We therefore developed a new method, in which bacterial DNA is recovered directly from the Gram-stained slide. Slides were Gram stained by traditional methodology (12). A clear glass slide was smeared with samples and treated with absolute methanol (Mallinckrodt, Hazelwood, Mo.), followed by heat fixation at 65°C and treatment with crystal violet solution (Remel, Lenexa, Kans.). The slide was further treated with Gram's iodine (Remel), decolorized with alcohol-acetone solution (3:1), and counterstained with safranin (Remel). Immersion oil was first removed from the slide with 100% xylene (J. T. Baker, Phillipsburg, N.J.) followed by rinsing in 100% ethanol. Then the material was scrapped with a straight razor blade, suspended in 50 μ l of Puregene-DNA hydration solution (Gentra, Minneapolis, Minn.), vortexed, and boiled for 10 min. For analysis of the blood isolate, material from both the BACTEC bottle and from colonies grown on solid medium was

used. Subsequent PCR was performed in a volume of 50 μ l containing 5 μ l of the template, 1.5 mM MgCl₂, 100 μ M each deoxynucleoside triphosphate (Roche Diagnostics Corporation, Indianapolis, Ind.), 1 U of *Taq* DNA polymerase (Roche Diagnostics Corporation), and 0.15 μ M (each) universal 16S rRNA primers 11E (5'-GAGGAAGGTGGGGATGACG-3') and 13B (5'-TCCGGGCCCTTGCATAAGTG-3') as described previously (15). After a denaturation step of 5 min at 94°C, PCR steps of 94°C for 60 s, 50°C for 60 s, and 72°C for 90 s were repeated 30 times in a Perkin-Elmer GeneAmp PCR system 9600 (Perkin-Elmer Applied Biosystems, Foster City, Calif.). Products were obtained from both sputum and blood (Fig. 1). These were purified with the QIAquick PCR purification kit (Qiagen Inc., Valencia, Calif.), and DNA sequencing

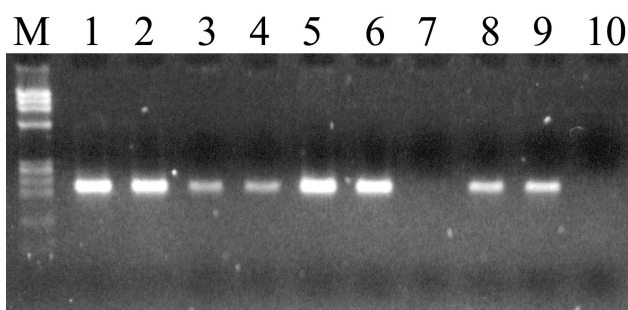


FIG. 1. 16S rRNA DNA amplification results. *E. coli* (ATCC 25922 lane 1), *Campylobacter jejuni* (ATCC 33291, lane 2), and *Staphylococcus aureus* (ATCC 25923, lanes 3 and 4) were used as controls. Bacterial DNA from the patient's blood was amplified from the BACTEC bottle (lane 5), from a plate colony (lane 6), and from Gram-stained respiratory specimens (BAL, lane 7; sputum samples, lanes 8 and 9). DNA was also recovered from one control sample (lane 4) after Gram staining and scraping. Lane 10 contained water, and lane M contained molecular size markers (ϕ X174-*Hae*III; New England Biolabs, Inc., Beverly, Mass.). PCR products (216 bp) were visualized by agarose gel electrophoresis (3% agarose gel; 1:2 [wt/wt] Seakem LE agarose-Nusieve GTG-agarose; FMC Bioproducts, Rockland, Maine) and DNA staining with ethidium bromide. Sequences obtained with universal primer PCR were confirmed for all control strains.

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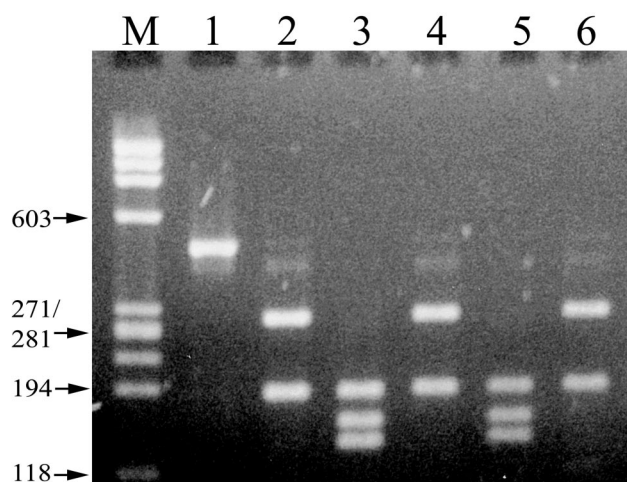


FIG. 2. Differentiation between *B. pertussis* and *B. holmesii* by *recA* gene amplification followed by digestion with *NciI*. The amplified segment of the *Bordetella recA* gene (483 bp) resulted in fragments of the following sizes after *NciI* digestion: *B. pertussis*, 295 and 188 bp; and *B. holmesii*, 188, 156, and 139 bp. Lanes: M, molecular size markers (in base pairs) (*phiX174-HaeIII*; New England Biolabs Inc.); 1, *B. holmesii* (ATCC strain, uncut); 2, *B. pertussis* (ATCC strain, plate colony); 3, *B. holmesii* (ATCC strain, plate colony); 4, *B. pertussis* (ATCC strain mixed with sputum, Gram stained); 5, *B. holmesii* (ATCC strain mixed with sputum, Gram stained); 6, patient's sputum Gram-stained sample. See the legend to Fig. 1 for a description of gel electrophoresis.

was performed on an Applied Biosystems ABI3100 sequencer (HHMI Biopolymer/W. M. Keck Foundation Biotechnology Resource Laboratory, Yale University School of Medicine). Subsequent comparison with the public database (GenBank) by BLAST (1) identified the blood isolate as *Helicobacter cinaedi* or *Helicobacter rappini*. Both organisms have been associated with bacteremias in immunocompromised patients (9, 10, 13, 16, 17). The PCR product of the respiratory isolate matched the 16S rRNA genes of both *Bordetella pertussis* and *Bordetella holmesii*, which are 99.5% homologous and identical in the amplified region (18).

In order to speciate the *Bordetella* respiratory isolate and to validate the result of the genotypical analysis, a discriminatory assay was developed in which a portion of the *recA* gene (6, 7), which contains a unique restriction enzyme site, was targeted.

Amplification of *B. pertussis* (ATCC 9340; American Type Culture Collection, Manassas, Va.) and *B. holmesii* (ATCC 51541) DNA as well as that from our patient's sputum Gram staining was carried out as described above, except that 3 mM MgCl₂ and newly designed specific primers (forward, 5'-CAA TACGCCTCCAAGCTGGG-3'; and reverse, 5'-TGATGTCTG AACTCGGCCTGC-3') were used, and the PCR steps (94°C for 30 s, 59°C for 30 s, and 72°C for 60 s) were repeated 45 times followed by a final elongation step at 72°C. Products were digested with *NciI* according to the manufacturer's recommendations (New England Biolabs, Inc., Beverly, Mass.). The two organisms could be easily distinguished because *B. holmesii* has two *NciI* sites, whereas *B. pertussis* and all other *Bordetella* spp. only have one *NciI* site within the amplified region. The sputum isolate was subsequently identified as *B. pertussis* (Fig. 2) and has been reported as an opportunistic lung pathogen in human immunodeficiency virus-infected patients (4, 5).

We next investigated whether the genotypical identification of organisms directly from clinical Gram stain preparations with universal primers is a feasible and reproducible method. The type of fixation (heat, 100% methanol, and 100% ethanol) and presence of dye remnants after Gram staining as described above do not interfere with the PCR. A detection limit of 1,500 CFU/ μ l of sputum (6 to 30 organisms per oil immersion field) was found, which is similar to other reports (14). For this finding, three random clinical sputum samples with no bacteria on Gram stain or in culture were pooled, spiked with defined amounts of *Escherichia coli* (ATCC 25922), fixed and Gram stained, and scraped off the slide as described above. Several randomly chosen clinical samples were tested, and when a predominant organism was visible on microscopy, its identification matched the cultured pathogen (Table 1).

Our method has several advantages over earlier described assays. Unlike previous reports, in which DNA is first extracted from sputum and species-specific primers are used (2, 11, 14, 19, 20), our assay uses DNA recovered directly from Gram-stained slides without extraction steps and utilizes universal primers, thus enabling the identification of a broad range of bacteria with a simple protocol. As shown, this can be achieved even in the presence of normal background resident flora because the number of PCR cycles is limited, thus allowing for competitive amplification of bacterial DNA. Since the quality

TABLE 1. Analysis of clinical sputum and wound specimens

Type of specimen	Phenotypical identification		Genotypical identification by using Gram-stained slides as template	
	Gram stain ^a	Culture	BLAST search	% Identity (identical/total bp)
Sputum	Scant WBC, moderate GNR, few mixed flora	Many <i>Pseudomonas aeruginosa</i> , moderate normal flora	<i>Pseudomonas</i> spp.	97 (113/116)
	Moderate WBC, many GNDC	Moderate <i>Branhamella catarrhalis</i>	<i>B. catarrhalis</i>	98 (150/152)
	Scant WBC, many GPCC	Many <i>S. aureus</i> , scant mixed flora	<i>S. aureus</i>	99 (153/154)
	Few WBC, moderate GNR	Many <i>Haemophilus influenzae</i> , scant <i>S. aureus</i>	<i>H. influenzae</i>	99 (162/163)
Wound	Many WBC, many GPCC	Many <i>S. aureus</i>	<i>S. aureus</i>	100 (156/156)
	Moderate GNR	Many <i>Enterobacter</i>	<i>Enterobacter</i> spp.	100 (154/154)

^a WBC, white blood cells; GNR, gram-negative rods; GNDC, gram-negative diplococci; GPCC, gram-positive cocci in clusters.

of the submitted specimen and the relative proportions of morphologically different microbes can be easily determined on Gram stains, suitable smears can be selected prior to DNA amplification. In addition, the Gram stain appearance of the predominant organism serves as an internal quality control after genotypical identification. As with phenotypical identification, the results of our assay have to be correlated with the clinical picture before treatment decisions are made, since neither method can reliably distinguish between colonization and infection.

Among the few reports in the English language literature describing recovery of nucleic acid from clinical specimens on glass slides (3, 8), none so far have described DNA amplification after recovery of bacteria from Gram-stained slides. Our method is fast and reliable and can be used as a tool in cases in which organisms are seen in abundance on clinical Gram-stained slides, but cannot be recovered in culture. The technique may be particularly useful when a diagnosis is sought retrospectively or after the original samples have been discarded.

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REFERENCES

- Altschul, S. F., W. Gish, W. Miller, E. W. Meyers, and D. J. Lipman. 1990. Basic local alignment search tool. *J. Mol. Biol.* **215**:403–410.
- Campbell, P. W., III, J. A. Phillips III, G. J. Heidecker, M. R. Krishnamani, R. Zahorchak, and T. L. Stull. 1995. Detection of *Pseudomonas (Burkholderia) cepacia* using PCR. *Pediatr. Pulmonol.* **20**:44–49.
- Chuaqui, R., K. Cole, M. Cuello, M. Silva, M. E. Quintana, and M. R. Emmert-Buck. 1999. Analysis of mRNA quality in freshly prepared and archival Papanicolaou samples. *Acta Cytol.* **43**:831–836.
- Colebunders, R., C. Vael, K. Blot, J. Van Meerbeeck, J. Van den Ende, and M. Ieven. 1994. *Bordetella pertussis* as a cause of chronic respiratory infection in an AIDS patient. *Eur. J. Clin. Microbiol. Infect. Dis.* **13**:313–315.
- Doebbeling, B. N., M. L. Feilmeier, and L. A. Herwaldt. 1990. Pertussis in an adult man infected with the human immunodeficiency virus. *J. Infect. Dis.* **161**:1296–1298.
- Favre, D., S. J. Cryz, Jr., and J. F. Viret. 1991. Cloning of the *recA* gene of *Bordetella pertussis* and characterization of its product. *Biochimie* **73**:235–244.
- Favre, D., and J. F. Viret. 1990. Nucleotide sequence of the *recA* gene of *Bordetella pertussis*. *Nucleic Acids Res.* **18**:4243.
- Fiel-Gan, M. D., C. F. Villamil, S. R. Mandavilli, M. E. Ludwig, and G. J. Tsongalis. 1999. Rapid detection of HSV from cytologic specimens collected into ThinPrep fixative. *Acta Cytol.* **43**:1034–1038.
- Gerrard, J., D. Alfredson, and I. Smith. 2001. Recurrent bacteremia and multifocal lower limb cellulitis due to *Helicobacter*-like organisms in a patient with X-linked hypogammaglobulinemia. *Clin. Infect. Dis.* **33**:E116–E118.
- Hung, C. C., P. R. Hsueh, M. Y. Chen, L. J. Teng, Y. C. Chen, K. T. Luh, and C. Y. Chuang. 1997. Bacteremia caused by *Helicobacter cinaedi* in an AIDS patient. *J. Formos. Med. Assoc.* **96**:558–560.
- Karpati, F., and J. Jonasson. 1996. Polymerase chain reaction for the detection of *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia* and *Burkholderia cepacia* in sputum of patients with cystic fibrosis. *Mol. Cell. Probes* **10**:397–403.
- Koneman, E. W. 1997. Color atlas and textbook of diagnostic microbiology, 5th ed. Lippincott, Philadelphia, Pa.
- Mammen, M. P., Jr., N. E. Aronson, W. J. Edenfield, and T. P. Endy. 1995. Recurrent *Helicobacter cinaedi* bacteremia in a patient infected with human immunodeficiency virus: case report. *Clin. Infect. Dis.* **21**:1055.
- McDowell, A., E. Mahenthiralingam, J. E. Moore, K. E. A. Dunbar, A. K. Webb, M. E. Dodd, S. L. Martin, B. C. Millar, C. J. Scott, M. Crowe, and J. S. Elborn. 2001. PCR-based detection and identification of *Burkholderia cepacia* complex pathogens in sputum from cystic fibrosis patients. *J. Clin. Microbiol.* **39**:4247–4255.
- Relman, D. A. 1993. Universal bacterial 16S rDNA amplification and sequencing, p. 489–495. In D. H. Persing, T. F. Smith, F. C. Tenover, and T. J. White (ed.), *Diagnostic molecular microbiology: principles and application*. American Society for Microbiology, Washington, D.C.
- Sullivan, A. K., M. R. Nelson, J. Walsh, and B. G. Gazzard. 1997. Recurrent *Helicobacter cinaedi* cellulitis and bacteraemia in a patient with HIV Infection. *Int. J. STD AIDS* **8**:59–60.
- Tee, W., A. Jenney, A. McPhee, A. Mijch, and M. Dyal-Smith. 2001. “*Helicobacter rappini*” isolates from 2 homosexual men. *Clin. Infect. Dis.* **33**:E8–E11.
- Weyant, R. S., D. G. Hollis, R. E. Weaver, M. F. M. Amin, A. G. Steigerwalt, S. P. O’Connor, A. M. Whitney, M. I. Daneshvar, C. W. Moss, and D. J. Brenner. 1995. *Bordetella holmesii* sp. nov., a new gram-negative species associated with septicemia. *J. Clin. Microbiol.* **33**:1–7.
- Whitby, P. W., K. B. Carter, J. L. Burns, J. A. Royall, J. J. LiPuma, and T. L. Stull. 2000. Identification and detection of *Stenotrophomonas maltophilia* by rRNA-directed PCR. *J. Clin. Microbiol.* **38**:4305–4309.
- Whitby, P. W., H. L. Dick, P. W. Campbell III, D. E. Tullis, A. Matlow, and T. L. Stull. 1998. Comparison of culture and PCR for detection of *Burkholderia cepacia* in sputum samples of patients with cystic fibrosis. *J. Clin. Microbiol.* **36**:1642–1645.