

Rapid Identification of *Actinobacillus actinomycetemcomitans*, *Haemophilus aphrophilus*, and *Haemophilus paraphrophilus* by Restriction Enzyme Analysis of PCR-Amplified 16S rRNA Genes

MARCELLO P. RIGGIO* AND ALAN LENNON

Infection and Immunity Research Group, University of Glasgow
Dental School, Glasgow G2 3JZ, United Kingdom

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Restriction enzyme analysis of PCR-amplified 16S rRNA genes was used to distinguish among clinical isolates of *Actinobacillus actinomycetemcomitans*, *Haemophilus aphrophilus*, and *Haemophilus paraphrophilus* which were originally identified by conventional phenotypic methods. This PCR-based method is a reliable and rapid alternative to conventional methods for identification of these bacterial species.

The closely related gram-negative species *Actinobacillus actinomycetemcomitans*, *Haemophilus aphrophilus*, and *Haemophilus paraphrophilus* are commonly found in the oral cavity of periodontally healthy humans (9, 11). *A. actinomycetemcomitans* has also been implicated in the pathogenesis of several forms of human periodontal disease, particularly localized juvenile periodontitis (25, 26) and severe adult periodontitis (4, 20). This association has come from the observance of high levels of *A. actinomycetemcomitans* in subgingival plaques from the periodontal pockets of individuals with active disease (21), and elevated titers of serum antibody to this organism have also been demonstrated in various forms of the disease (8, 13, 16). *H. aphrophilus* and *H. paraphrophilus* have also been isolated from subgingival plaque (11), but because they are generally found in much smaller numbers than *A. actinomycetemcomitans*, they are regarded as playing no significant role in the pathogenesis of periodontal disease.

Differentiating among these species by conventional culture methods has previously proven to be a difficult procedure, mainly due to their similar growth characteristics, colony morphology, and microscopic appearance. *A. actinomycetemcomitans* has been differentiated from the other two species by a combination of several tests. A selective medium for growth of *A. actinomycetemcomitans*, namely, tryptic soy bacitracin vancomycin (TSBV) agar, has been widely used in combination with a characteristic star-shaped morphology and a positive catalase test for presumptive identification of this organism (19). More accurate identification of *A. actinomycetemcomitans* has been achieved by the use of phenotypic tests, particularly fermentation of specific sugars (18), which are able to discriminate it from *H. aphrophilus* and *H. paraphrophilus*. However, phenotypic tests can sometimes be inconclusive, since strains of *A. actinomycetemcomitans* which are catalase negative or which have unexpected sugar fermentation characteristics have been reported (22, 23). Due to their phenotypic similarity, the only method available for distinguishing between *H. aphrophilus* and *H. paraphrophilus* is based upon

the requirement for V factor for the growth of *H. paraphrophilus* (10).

More recently, molecular technique-based tests have been developed for discriminating between *A. actinomycetemcomitans* and *H. aphrophilus* or *H. paraphrophilus*. These include the use of species-specific synthetic-oligonucleotide probes and cloned-gene probes for use in DNA-DNA hybridization assays (5, 7, 24) and electrophoretic analysis of 23S rRNA (14). However, adequate discrimination between *H. aphrophilus* and *H. paraphrophilus* has yet to be achieved by molecular techniques.

In this study we report the development of a rapid, molecular-based method for distinguishing among *A. actinomycetemcomitans*, *H. aphrophilus*, and *H. paraphrophilus* which is based on restriction enzyme analysis of PCR-amplified 16S rRNA genes of these species. The method was applied to stored clinical isolates which had been classified as either *A. actinomycetemcomitans*, *H. aphrophilus*, or *H. paraphrophilus* by conventional culture methods and sugar fermentation tests. A total of 45 isolates, of which 20 were categorized as *A. actinomycetemcomitans*, 20 were categorized as *H. aphrophilus*, and 5 were categorized as *H. paraphrophilus*, were examined. An additional two isolates tentatively identified as *A. actinomycetemcomitans* on the basis of characteristic sugar fermentation profiles, but which were catalase negative, were also examined. Isolates had originally been obtained from the culture of subgingival plaque samples from patients with adult periodontitis or gingivitis who were being treated at Glasgow Dental Hospital. Samples were cultured by inoculation onto TSBV agar (19) and chocolate agar (11) plates and were incubated in an atmosphere of 5% CO₂–95% air at 37°C for 3 days. For isolation of *A. actinomycetemcomitans* and *H. aphrophilus*, colonies from TSBV agar plates with characteristic colony morphology were subcultured to Columbia agar plates (Life Technologies Ltd., Paisley, Scotland) supplemented with 7.5% sterile defibrinated horse blood and were incubated as described above, whereas colonies from chocolate agar plates which were suspected of being *H. paraphrophilus* were subcultured to chocolate agar plates and were also incubated as described above. Colonies which contained gram-negative coccobacilli which required carbon dioxide for growth, which were catalase positive, and which fermented glucose and maltose but not lactose, sucrose, salicin, or arabinose were recorded as *A. actinomycetemcomitans*; colonies which were catalase nega-

* Corresponding author. Mailing address: Level 9, Glasgow Dental Hospital and School, 378 Sauchiehall St., Glasgow G2 3JZ, Scotland, United Kingdom. Phone: (0141) 211 9742. Fax: (0141) 353 1593. E-mail: m.riggio@dental.gla.ac.uk.

tive and which additionally fermented sucrose and lactose were identified as *H. aphrophilus*. Colonies isolated from chocolate agar plates which demonstrated V-factor dependence for growth as determined by the Microring XV system (Medical Wire and Equipment Co. Ltd., Corsham, England), which were catalase negative, and which had the sugar fermentation characteristics described above for *H. aphrophilus* were identified as *H. paraphrophilus*. Although *H. paraphrophilus* is V factor dependent for growth, *H. aphrophilus* is not X factor dependent. Crude DNA extracts were prepared from each isolate by inoculation of two or three loopfuls of bacteria from the surface of an agar plate into 200 μ l of sterile molecular-biology-grade water, boiling for 10 min, removal of cell debris by centrifugation, and retention of the supernatant.

PCR amplification (15) was carried out with each DNA extract. The primers used for amplification targeted conserved regions of the 16S rRNA gene and were designed to amplify DNA from most bacterial species. The primers used were 5'-TCC TAC GGG AGG CAG CAG-3' (16S1; *Escherichia coli* positions 340 to 357) and 5'-CCC GGG AAC GTA TTC ACC G-3' (16S2; *E. coli* positions 1387 to 1369), which give an expected amplification product of 1,045 bp. PCR was carried out in a total volume of 100 μ l, consisting of 5 μ l of DNA extract and 95 μ l of PCR mixture containing 1 \times PCR buffer (10 mM Tris-HCl [pH 8.8], 1.5 mM MgCl₂, 50 mM KCl, 0.1% Triton X-100), 2.0 U of Dynazyme I DNA polymerase (Flowgen Instruments Ltd., Lichfield, England), each deoxynucleotide triphosphate at 0.2 mM, and each primer at 0.2 μ M. Negative PCR controls contained 5 μ l of sterile water instead of template DNA. PCR amplification was carried out in an OmniGene thermal cycler (Hybaid Ltd., Teddington, England). An initial denaturation step at 94°C for 5 min was carried out, followed by 30 cycles of denaturation at 94°C for 1 min, primer annealing at 60°C for 1 min, and primer extension at 72°C for 1.5 min, and a final extension step at 72°C for 10 min. Ten microliters of each PCR product was electrophoresed on a 2% agarose gel. Gels were stained with ethidium bromide (0.5 μ g/ml), and DNA was visualized under UV light.

PCR products were purified with the Wizard PCR Purification Kit (Promega Corporation, Southampton, England) according to the manufacturer's instructions. Approximately 0.5 μ g of purified PCR product was digested in a total volume of 20 μ l with 10 U (each) of the restriction enzymes *Hha*I and *Hin*FI (Life Technologies) at 37°C for 3 h. Restriction fragments were visualized by gel electrophoresis as described above.

The 16S rRNA gene was successfully amplified from all isolates tested, as demonstrated by the appearance of a 1,045-bp PCR product (data not shown). The expected sizes of the restriction fragments (at least 100 bp long) generated by digestion of PCR products from each species with *Hha*I are as follows: *A. actinomycetemcomitans*, 735 and 279 bp; *H. aphrophilus*, 634, 279, and 100 bp; and *H. paraphrophilus*, 481, 279, and 253 bp. For digestion with *Hin*FI, the sizes of expected restriction fragments for each species are as follows: *A. actinomycetemcomitans*, 937 bp; *H. aphrophilus*, 661 and 275 bp; and *H. paraphrophilus*, 791 and 145 bp. Typical restriction patterns obtained following digestion of PCR products with *Hha*I and *Hin*FI are shown in Fig. 1 and are in accordance with the restriction profiles expected. Both *Hha*I and *Hin*FI give distinct restriction patterns for each of the three species. For all isolates tested which were adequately categorized by conventional methods (20 *A. actinomycetemcomitans*, 20 *H. aphrophilus*, and 5 *H. paraphrophilus* isolates) species identification by the PCR-based method correlated perfectly with the results obtained by conventional identification methods. Additionally, the two

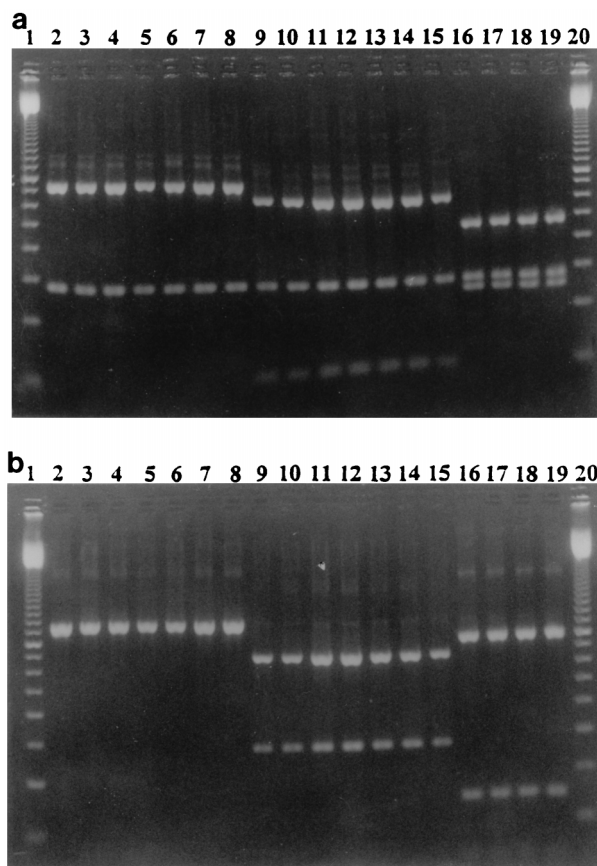


FIG. 1. Electrophoresis on 2% agarose gels of restriction fragments obtained following digestion of 16S rRNA PCR products with restriction enzymes *Hha*I (a) and *Hin*FI (b). Lanes: 1 and 20, 100-bp DNA ladder; 2, *A. actinomycetemcomitans* ATCC 29524; 3 to 8, *A. actinomycetemcomitans* isolates; 9, *H. aphrophilus* ATCC 33389; 10 to 15, *H. aphrophilus* isolates; 16, *H. paraphrophilus* ATCC 29241; and 17 to 19, *H. paraphrophilus* isolates.

catalase-negative isolates tentatively identified as *A. actinomycetemcomitans* on the basis of their sugar fermentation profiles were confirmed as such by the PCR-based method.

Conventional methods for identifying *A. actinomycetemcomitans*, *H. aphrophilus*, and *H. paraphrophilus* are reliant upon examination of the phenotypic characteristics of each species. As we have shown in this study, these methods are generally reliable, as shown by correlation of the results obtained by conventional identification methods with those obtained by the PCR-based method. However, conventional methods for species identification are time-consuming and labor-intensive. Undoubtedly, strains which have variable expression of phenotypic characteristics may arise. For example, strains of *A. actinomycetemcomitans* which are catalase negative or have unusual sugar fermentation profiles have been isolated (22, 23). In such cases, conventional methods would be unable to definitively identify these phenotypically variable strains; however, we have demonstrated in this study the utility of our PCR-based method for the definitive identification of two catalase-negative isolates as *A. actinomycetemcomitans*.

The misidentification of *H. paraphrophilus* by conventional identification methods has been reported (3). Such problems can be overcome with the use of the PCR-based identification method we have presented in this study, which is the first to demonstrate positive identification of *H. paraphrophilus* by mo-

lecular methods. Another simple and rapid method for distinguishing between *A. actinomycetemcomitans* and *H. aphrophilus* which is based upon the observation that the 23S rRNA molecule is intact in *H. aphrophilus* but split into two smaller units in *A. actinomycetemcomitans* has been reported (14). However, this method is unable to discriminate between *H. aphrophilus* and *H. paraphrophilus*. Oligonucleotide hybridization probes specific for *A. actinomycetemcomitans* and *H. aphrophilus* have also been developed (7).

Some controversy exists as to whether *H. aphrophilus* and *H. paraphrophilus* should be regarded as separate species or as a single species. Multivariate analysis of enzyme data (12) and of carbohydrate data from lipopolysaccharides (1) suggests classification as two distinct species. Conversely, multilocus enzyme electrophoresis (2), comparison of 16S rRNA genes (6), and ribotyping with a 16S-23S rRNA probe (17) suggest that they are a single species.

In conclusion, we have developed a rapid, accurate, and specific method for distinguishing among the closely related species *A. actinomycetemcomitans*, *H. aphrophilus*, and *H. paraphrophilus*. We suggest the use of this method as an alternative and improved confirmatory procedure to conventional phenotypic tests for species-level identification of clinical isolates initially identified by culture methods as possibly being *A. actinomycetemcomitans*, *H. aphrophilus*, or *H. paraphrophilus*. The assay is simpler, cheaper, and more rapid than conventional phenotypic identification methods and provides unequivocal results, since it can identify phenotypically variable strains. The assay is useful in a clinical context, since it is important to be able to unequivocally distinguish the more pathogenic *A. actinomycetemcomitans* from *H. aphrophilus* and *H. paraphrophilus*, and it has the added versatility of being able to distinguish between *H. aphrophilus* and *H. paraphrophilus*. It is important to obtain and identify clinical isolates which could be used in further studies, such as antibiotic sensitivity testing and genetic typing of isolated strains. The features of our assay render it useful in both clinical and reference laboratory settings.

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