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Rapid Presumptive Identification of Black-Pigmented Gram-Negative Anaerobic Bacteria by Using 4-Methylumbelliferone Derivatives

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A rapid method for presumptive identification of black-pigmented gram-negative anaerobic rods was developed. Using filter paper spot tests for indole production, sialidase, α-glucosidase, β-glucosidase, α-fucosidase, and trypsinlike enzyme activities, 100% of *Porphyromonas gingivalis*, *Prevotella intermedia*, and *Bacteroides levii* and 89% of *Prevotella corporis* isolates were correctly identified to the species level. *Porphyromonas asaccharolytica* and *Porphyromonas endodontalis* could not be differentiated from each other but could be distinguished from all other species tested. Similarly, *Prevotella denticola*, *Prevotella loescheii*, and *Prevotella melaninogenica* could not be differentiated from each other. The methods described are based on 4-methylumbelliferone derivatives of the various substrates and are simple to perform, rapid (<15 min), and applicable to difficult-to-cultivate anaerobic rods.

Black-pigmented anaerobic bacilli are important pathogens in the oral cavity, in the female genital tract, and in other body sites. Isolation and identification to the species level is expensive, is time-consuming, and requires a highly trained technologist and specialized equipment. Identification of these organisms to the species level is desirable in order to clarify the role of *Porphyromonas* and *Prevotella* species in human infections and in some instances to help to identify whether the source of infection is oral or genital. The most reliable methods for identification of these microorganisms require extensive biochemical tests and analysis of metabolic products by gas-liquid chromatography (5). Over the past several years substantial progress has been made in the development of DNA-based probes for the identification of anaerobes (4, 10, 12). However, such technologies are not readily available to most clinical microbiologists or to researchers in other areas. The specificity of DNA probes limits their application to only those species for which probes have been developed. Identification methods based on preformed enzymes have gained acceptance and are available from commercial sources (2, 3, 6, 14, 15, 20). These rapid tests are expensive, and the species identification is limited by the data base of the manufacturer.

The taxonomy of the black-pigmented anaerobic rods (formerly the genus *Bacteroides*) is currently under revision (16–18). The recognized species include *Prevotella intermedia*, *Prevotella denticola*, *Prevotella corporis*, *Prevotella loescheii*, and *Prevotella melaninogenica*, as well as *Porphyromonas asaccharolytica*, *Porphyromonas gingivalis*, and *porphyromonas endodontalis* (16–18). These organisms are all inhabitants of the human mucosal surfaces and represent significant infectious agents (8). *Bacteroides levii* and *Bacteroides macacae*, the only black-pigmented anaerobic rods remaining in the genus *Bacteroides*, are isolated only from nonhuman sources.

The use of 2′-(4-methylumbelliferyl)α-D-*N*-acetylneuraminic acid to detect bacterial sialidase activity in a rapid filter paper spot test among *Actinomyces* species and gram-negative anaerobic and capnophilic species has been reported (9, 11). These studies demonstrated that sialidase activity was often species specific. Sialidase activity was detected in 100% of all *P. denticola*, *P. loescheii*, *P. melaninogenica*, *B. levii*, and *P. gingivalis* isolates tested but not in any *P. corporis*, *P. intermedia*, *P. asaccharolytica*, or *P. endodontalis* isolates. Using the spot sialidase test in conjunction with the spot indole test, we observed that these tests could be useful in the initial grouping of clinical isolates. The studies reported here were undertaken to extend these initial observations for the purpose of obtaining additional rapid methods for the presumptive identification of these organisms.

MATERIALS AND METHODS

Microorganisms and culture condition. The reference organisms obtained from the American Type Culture Collection (ATCC) were *P. asaccharolytica* (*Bacteroides asaccharolyticus*) ATCC 25260, *P. endodontalis* ATCC 35406, *P. gingivalis* ATCC 33277, *P. corporis* (*Bacteroides corporis*) ATCC 33547, *P. denticola* ATCC 33185, *P. intermedia* ATCC 25611 and ATCC 33563, *P. loescheii* ATCC 15930, *P. melaninogenica* ATCC 25845, and *B. levii* ATCC 29147. Six field strains of *P. endodontalis* were provided by A. J. van Winkelhoff, Amsterdam, The Netherlands. All other organisms were isolated from oral or genital clinical specimens as previously described (7, 12). Bacteria were maintained anaerobically at 37°C on prereduced heart infusion agar (Difco Laboratories, Detroit, Mich.) containing 5% defibrinated sheep blood. Some *P. gingivalis* and *P. endodontalis* isolates required coculture with *Staphylococcus aureus* on the agar plate for optimal growth. Organisms were tested for enzyme activity after 2 to 3 days of culture or when adequate growth had occurred.

Assays. For the spot trypsinlike enzymed assay, 10 mg of *N*-carbobenzoxy-L-arginine 7-amido 4-methylcoumarin hydrochloride (Sigma) was dissolved in 4.16 ml of dimethyl sulfoxide. The substrate was stored at 4°C until needed. For assays, 100 μl of the *N*-carbobenzoxy-L-arginine 7-amido 4-methylcoumarin hydrochloride in dimethyl sulfoxide was mixed in with 100 μl of 0.1 M Tris buffer, pH 8.0, and used to soak a filter paper strip.

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TABLE 1. Enzyme activities of black-pigmented anaerobic rods observed with rapid filter paper spot tests

Organism (no. of isolates tested)	% Isolates giving positive reaction for following tests:					
	Indole	Sialidase	α-Glucosidase	β-Glucosidase	α-Fucosidase	Trypsinlike enzyme[a]
Porphyromonas asaccharolytica (18)	100	0	0	0	22	0
P. endodontalis (8)	100	0	0	0	0	0
P. gingivalis[b] (29)	100	100	0	0	0	100
Prevotella intermedia (40)	100	0	100	0	85	0
P. corporis (9)	0	11	100	0	0	0
P. denticola (12)	0	100	100	58	100	ND
P. loescheii (10)	0	100	100	100	100	ND
P. melaninogenica (13)	0	100	100	0	100	ND
Bacteroides levii[c] (5)	0	100	40	0	0	0

[a] ND, not done.
[b] Data include 16 isolates obtained from *Macaca fasicularis*.
[c] Data include four isolates from human beings which most closely resemble *B. levii*.

Substrates for glucosidase assays used in these studies, 4-methyllumbelliferyl-α-D-glucoside and 4-methylumbelliferyl-β-D-glucoside, were suspended in distilled water to a concentration of 4 mM. 4-Methylumbelliferyl-α-fucoside was dissolved in distilled water to a concentration of 1 mM. Most substrates formed a suspension rather than a true solution. When this was observed, the suspensions were allowed to stand for 5 to 10 min to allow particulate material to settle out of solution; then the supernatant was decanted and dispensed prior to freezing. Substrates were dispensed in 180-μl volumes and stored at −20°C until used. For use, 20 μl of 1 M sodium phosphate buffer, pH 7.0, was added and mixed in with the substrate, and the resulting solution was used to soak a Whatman no. 2 filter paper strip.

Bacterial sialidase activity and the spot indole (*p*-dimethlyaminocinnamaldehyde method) tests were performed as described previously (9, 11, 19).

The filter paper spot test was performed by smearing a loop of bacterial growth onto the filter paper strips (~6 by 60 mm) containing the substrates and incubating at 37°C for 15 min. Usually, 12 to 20 isolates could be tested on a single strip. The filter strips were examined after 15 min under a long-wavelength (365 nm) hand-held mineral lamp. Enzymatic activity was observed as a fluorescent blue spot on the filter paper.

RESULTS AND DISCUSSION

The enzyme activities detected for the indicated species are presented in Table 1.

The results for the species of *Porphyromonas* (*P. gingivalis*, *P. asaccharolytica*, and *P. endodontalis*) and the possibly closely related organism *B. levii* (17) were distinct enough to allow differentiation of these organisms from the other species tested. *Porphyromonas* spp. lacked glucosidase, which is consistent with previous reports (20). *P. gingivalis* was easily recognized by the uniform presence of trypsinlike enzyme and sialidase activity, as well as indole production. The differentiation of *P. endodontalis* and *P. asaccharolytica* was difficult, except for the 21% of *P. asaccharolytica* isolates positive for α-fucosidase activity. While previous studies have reported that most (5 of 6 [3] and 8 of 8 [20]) *P. asaccharolytica* tested positive for this characteristic, a much larger number of strains were included in the present report. More sophisticated tests would be required to distinguish *P. asaccharolytica* from *P. endodontalis*, e.g., sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), DNA-DNA homology, or growth requirements (8, 21). Since it appears that *P. asaccharolytica* is very rarely found in the oral cavity and *P. endodontalis* has not been isolated from sources other than the oral cavity, the site of isolation could be used to differentiate these organisms.

The use of the identification scheme presented in Table 2 allows presumptive identification of *P. intermedia* and *P. corporis*; all *P. intermedia* isolates were correctly identified by the spot test, as were 89% of the *P. corporis* isolates. *P. intermedia* contains at least two genetically distinct species (12) represented by strains ATCC 25611 (type I), ATCC 33563 (type II), and one recently described, ATCC 49046 serotype C (13). In a previous study we reported that 12% of our *P. intermedia* isolates failed to react with whole-cell DNA probes to either *P. intermedia* type I or II (12). These

TABLE 2. Scheme for presumptive identification of black-pigmented anaerobic rods using spot tests

Species	Reaction[a] with following tests:					Other characteristics
	Indole	Sialidase	α-Glucosidase	α-Fucosidase	β-Glucosidase	
Porphyromonas gingivalis	+	+	−	−	−	Trypsinlike enzyme
P. asaccharolytica	+	−	−	V	−	Nonoral sites
P. endodontalis	+	−	−	−	−	Oral cavity
Prevotella intermedia	+	−	+	V	−	
P. loeschii	−	+	+	+	+	Acid from cellobiose, minor acetic acid
P. denticola	−	+	+	+	V	No acid from cellobiose, Acid from esculin
P. melanionogenica	−	+	+	+	−	No acid from cellobiose or esculin
P. corporis	−	V	+	−	−	
Bacteroides levii	−	+	V	−	−	

[a] +, 90 to 100% of strains positive; −, 90 to 100% of strains negative; V, variable (11 to 89% positive.)

strains failed to react with RNA probes specific for *P. intermedia* types I and II. Since the serotype C strain hybridized with the *P. intermedia* type I probe, these results suggest there are three genetically distinct groups within this species. The ATCC strain of *P. intermedia* serotype C gave reactions identical to those observed for the other two DNA homology groups. We were unable to differentiate these organisms by spot test. In this study we used six isolates of *P. intermedia* isolated from human vagina; these isolates gave sugar fermentation patterns and gas-liquid chromatography patterns consistent with an identification of *P. intermedia*. However, these isolates all failed to give a positive α-fucosidase reaction. These isolates were tested with oligonucleotide probes specific for *P. intermedia* types (I and II) and failed to hybridize with the probes, indicating that they are not genetically *P. intermedia* and most likely represent an undescribed species.

The spot tests were useful for presumptive identification of *P. denticola*, *P. loescheii*, and *P. melaninogenica* to group but not to species level.

The ATCC type strain of *B. macacae* was positive for sialidase, α-glucosidase, α-fucosidase, and trypsinlike enzyme and negative for β-glucosidase. These results should be considered tentative since only one strain was tested and the results were difficult to interpret. However, this organism differs from the others in being catalase positive.

The rapid spot test herein reported provides an excellent means of presumptive identification of black-pigmented anaerobic rods, while affording several advantages over other rapid identification methods. Several identification systems based on preformed enzymes are commercially available, such as API An-Ident (Analytab Products, Inc., Plainview, N.Y.), the RapID-ANA (Innovative Diagnostic Systems, Inc., Atlanta, Ga.), and the Vitek ANI card (Vitek Systems, Hazelwood, Mo.) (2, 14, 15). They are designed for use on a wide range of different bacteria and therefore contain many enzyme tests which are not needed for the specific groups of microorganisms. Also, these test kits are somewhat expensive in comparison to the rapid spot reagents used here, which cost from $0.20 to $1.75 per battery of tests, depending on the number of isolates tested.

The substrates used by many commercial test systems are β-naphthylamide derivatives which are approximately 1,000-fold-less sensitive than the fluorescent compounds used in the present study (1). The increased sensitivity in detecting 4-methylumbelliferone results in much shorter incubation times (15 min versus 2 to 4 h). Cells are more concentrated on the test strip, which contributes to the rapid detection of enzyme activity in the spot test. We have observed some reaction variability when enzyme activities were measured with the naphthylamide derivatives. However, variable results were seldom observed with the spot test. Another advantage of the present method is that growth in peptone yeast with or without glucose broth is not required. Many of these nutritionally fastidious isolates are difficult to cultivate in liquid media, making sugar fermentation patterns difficult to interpret. Three *P. intermedia* isolates were initially presumptively identified as *P. asaccharolytica* because they failed to produce acid from glucose, lactose, or sucrose or to demonstrate indole production in the tube test. In all three cases, growth in the broth was very light. Gas-liquid chromatography analysis of PYG broth (5) demonstrated major succinic and acetic acid peaks with minor isovaleric and isobutyric peaks, consistent with an identification of *P. intermedia*, the identification made by using the scheme in Table 2. Thus, the use of these spot tests may be especially useful for organisms which are difficult to cultivate in broth media. One disadvantage of the proposed method, which is shared by other standard methods, is the difficulty in identifying isolates which fail to express black pigmentation. This proposed identification scheme should be applied only to those anaerobic rods which are black pigmented or fluoresce brick red. The method has not been defined for nonpigmented organisms.

The filter paper spot test was found to be a valuable tool for rapid presumptive identification of black-pigmented-gram-negative anaerobic bacteria.

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