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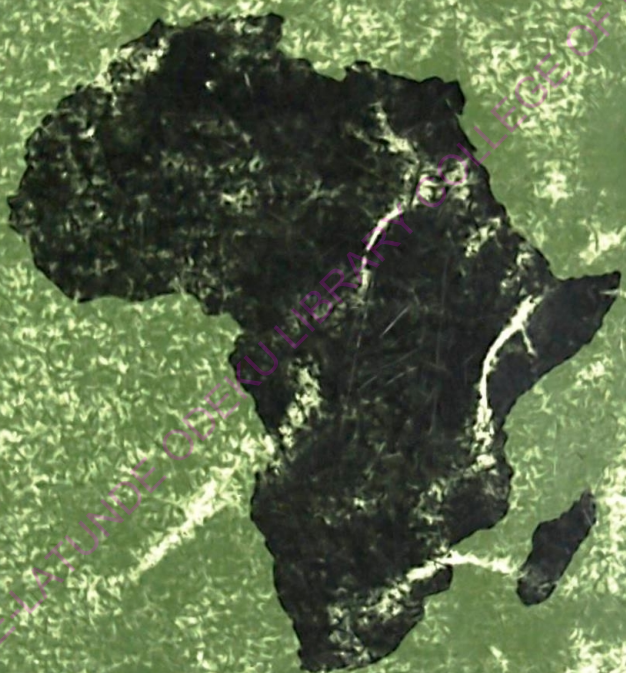

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Rapid presumptive identification of human black pigmented *Bacteroides* species

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Summary

A simple and reliable technique is described for the rapid presumptive identification of black pigmented *Bacteroides* species of human origin. This method involved a microtitration technique that detected the hydrolysis of specific chromogenic enzyme substrates and haemagglutination of sheep erythrocytes. Pure cultures of black pigmented *Bacteroides* strains, representing the eight human species, were successfully differentiated and identified within 4 h by the identification scheme developed with this method. This is a highly reproducible method and the scheme should be useful in laboratories lacking the sophisticated equipment often needed for the identification of black pigmented *Bacteroides*.

Résumé

Une technique simple et assuré est décrite pour l'identification présomptueuse rapide de *Bacteroides* pigmenté noir (BPB), une espèce d'origine humain. Cette méthode a comporté une technique de microtitration qui a détecté l'hydrolyse de substrats spécifiques et l'haemagglutination des cellules rouges de sang du mouton.

Les cultures purs de la race *Bacteroides* pigmenté noir et qui représentent huit espèces humaines ont eu succès à différencier et identifier pendant 4 heures par la manière d'identification développé avec cette méthode. C'est une méthode qu'on peut reproduit et il faut l'utiliser dans les laboratoires quit manquent l'euipement compliqué qu'on emploie à l'identification de BPB.

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Introduction

Black pigmented *Bacteroides* (BPB) was first isolated from humans by Oliver and Wherry [1] and was classified as *B. melaninogenicus*. This original species has since undergone a series of taxonomical changes and now comprises ten species, including *B. melaninogenicus*, *B. intermedius*, *B. denticola*, *B. corporis*, *B. loescheii*, *B. asaccharolyticus*, *B. gingivalis*, *B. endodontalis*, *B. levii* and *B. macacae*.

B. melaninogenicus as originally described by Oliver and Wherry [1] was initially separated by Holdeman and Moore [2] into three subspecies, *B. melaninogenicus* ss *intermedius* and *B. melaninogenicus* ss *asaccharolyticus*, based on sugar fermentation reactions reported earlier [3]. Since then, recent advances in the taxonomy of BPB have been based on nucleic acid studies and chromatographic analysis of volatile and non-volatile acid end-products of amino acid and glucose metabolism [4–8].

However, the facilities and expertise required for these procedures are not readily available in several laboratories in the third world, particularly in tropical Africa, and identification of BPB is not usually undertaken when they are isolated from clinical specimens. This problem is evident in several published reports, which conveniently refer to these species as the '*B. melaninogenicus* group', the 'pigmented *Bacteroides*' or 'black pigmented *Bacteroides*' without further characterization and speciation.

In this paper we report a simple, reproducible technique which was developed for the rapid presumptive speciation of human BPB.

Materials and methods

Organisms

Black pigmented *Bacteroides* species used for developing and evaluating this scheme are listed in Table 1. They represent reference strains (ATCC: American Type Culture Collection, Rockville, MD, U.S.A.), and clinical isolates previously characterized and identified according to standard methods [9,10].

Media

Media used in the development of this scheme were blood agar, enriched trypticase soya agar (ETSA) [11], anaerobic blood agar [12] and anaerobic blood agar (with supplements and different concentrations of sheep blood, e.g. 5%, 7% and 9%).

Table 1. Reference and clinical black pigmented *Bacteroides* species used for developing and evaluating scheme

Bacteria	Strain
B. gingivalis	ATCC 33277
B. gingivalis	CS 43*
B. gingivalis	Barbs†
B. gingivalis	Nig, 2, 3, 6, 7, 8, 9, 10
B. asaccharolyticus	ATCC 25260
B. asaccharolyticus	Nig 5, 6
B. intermedius	ATCC 25611
B. intermedius	CS34*, Nig 14, 14B
B. melaninogenicus	ATCC 25843
B. melaninogenicus	CS*
B. denticola	ATCC 33185
B. loescheii	ATCC 33547
B. corporis	ATCC 33547
B. endodontalis	ATCC 35406

ATCC = American Type and Culture Collection, Rockville, MD, U.S.A.; Nig = Clinical strains isolated from oro-dental and other infections in Nigeria.
*Courtesy of Dr S. Martins and Ms L. Bobo, Microbiology Deparment, University of Maryland Dental School, Baltimore, MD, U.S.A.
†Courtesy of Dr B. E. Laughon, Infectious Diseases Laboratory, Johns Hopkins Hospital, Baltimore, MD, U.S.A.

Antibiotic discs

Antibiotic discs (BBL) used were kanamycin (1000 μg) and metronidazole (4 μg).

Reagents and enzyme substrates

Reagents employed in this scheme included dimethylsulphoxide (DMSO), para-dimethylaminocinimaldehyde (DMCA), xylene, mercapto-ethanol and magnesium chloride. The specific enzyme substrates used and their concentrations were:

1. L-tryptophan (Sigma) 0.43% w/v
2. P-nitrophenyl-α-D-glucopyranoside (Sigma) 0.15% w/v
3. P-nitrophenyl-β-D-glucopyranoside (Sigma) 0.15% w/v
4. P-nitrophenyl-α-L-fucopyranoside (Sigma) 0.20% w/v
5. P-nitrophenyl-N-acetyl-β-D-glucosaminide (Sigma) 0.28% w/v
6. N-α-benzyl-DL-arginine-P-nitroanilide HCl (BAPNA; Sigma) 0.40% w/v

Enzyme substrate solutions, with the exception of BAPNA, were prepared by dissolving known weights of powdered enzyme substrate in appropriate volume of deionized distilled water to attain the desired concentration. These were filter-sterilized and stored at 4°C until used. BAPNA solution was prepared by dissolving 44 mg of BAPNA substrate in 1 ml of DMSO and was stored at 4°C. Before use, this solution was diluted 1 : 100 in trypsin activator solution and then filter-sterilized. Trypsin activator solution consists of 0.1 M Tris, 0.1% (w/v) magnesium chloride and 2% mercapto-ethanol (pH 8.4).

Preparation of 3% sheep blood solution

A 3% suspension of sheep erythrocytes was prepared by centrifuging 10 ml of defibrinated sheep blood at 200 *g* for 5 min, the supernatant was discarded and the sediment erythrocytes were washed twice in phosphate buffered saline — PBS (pH 7.2). The resulting sediment was then diluted in PBS to give a 3% (v/v) suspension.

Identification procedure

Identification of the BPB was accomplished by the various enzymatic activities and haemagglutination of sheep erythrocytes; 50 µl of each enzyme substrate solution listed above and 50 µl 3% sheep blood were dispensed into round-bottom wells in labelled microtitre plates and 50 µl of a dense suspension of pure culture of BPB species was added. The mixture was then incubated in air for 4 h at 37°C. Sterile PBS was added to each substrate solution in a separate row of wells as a control.

Hydrolysis of any chromogenic enzyme substrate by specific enzyme produced by the bacteria resulted in the release of a yellow nitrophenol compound or, in the case of BAPNA, a yellow p-nitroanilide compound, which was considered positive when produced within 4 h. Production of indole from tryptophan was detected by initially adding a drop of xylene to the test bacterial suspension, which extracted and concentrated any indole present. The presence of indole was then detected by adding a drop of 1% DMCA in 10% (v/v) hydrochloric acid. Formation of a blue colour was regarded as positive for indole and any other colour change was regarded as negative. The enzyme activity detected by specific substrates can be seen in Table 2.

Qualitative haemagglutination activity in wells was considered negative if erythrocytes and bacteria formed a closely-packed mass at the bottom of the well, and positive if they formed a smooth mat with crenated edges.

Results

The enzymatic and haemagglutinating activity of the different black pigmented *Bacteroides* species are presented in Table 3.

The asaccharolytic species, i.e. *B. asaccharolyticus*, *B. gingivalis* and *B. endodontalis*, did not hydrolyse P-nitrophenyl-α-D-glucopyranoside and P-nitrophenyl-β-D-glucopy-

Table 2. Enzymatic activity detected by specific enzyme substrates

Substrate	Enzyme detected
P-nitrophenyl-α-D-glucopyranoside	α-D-glucosidase
P-nitrophenyl-β-D-glucopyranoside	β-D-glucosidase
P-nitrophenyl-α-L-fucopyranoside	α-fucosidase
P-nitrophenyl-N-acetyl-β-D-glucosaminide	N-acetyl-β-D-glucosaminidase
N-α-benzoyl-DL-arginine-P-nitroanilide HCl	Trypsin
Tryptophan	Tryptophanase

Table 3. Enzymatic and haemagglutinating activities of human black pigmented *Bacteroides* species

Species	Indole	α-glu	β-glu	α-fuc	N-acetyl	Trypsin	HA
B. gingivalis	+	−	−	−	−	+	+
B. asaccharolyticus	+	−	−	+	−	−	−
B. endodontalis	+	−	−	−	−	−	−
B. melaninogenicus	−	+	−	+	+	−	−
B. intermedius	+	+	−	−	−	−	−
B. loescheii	−	+	+	+	+	−	−
B. denticola	−	+	−	−	+	−	−
B. corporis	−	+	−	−	−	−	−

α-glu = α-D-glucosidase; β-glu = β-D-glucosidase; α-fuc = α-fucosidase; N-acetyl = N-acetyl-β-D-glucosaminidase; trypsin = trypsin-like activity; HA = haemagglutination of 3% (v/v) sheep red blood cells.

ranoside, but all of them produced indole from tryptophan. *B. asaccharolyticus* hydrolysed P-nitrophenyl-L-α-fucopyranoside, while only *B. gingivalis* haemagglutinated sheep erythrocytes. Indeed, demonstration of trypsin-like activity and haemagglutination of sheep erythrocytes were peculiar only to *B. gingivalis* among the BPB species. *B. endodontalis* was differentiated by its inability to hydrolyse any of the enzyme substrates and lack of demonstrable trypsin-like activity.

The saccharolytic species, i.e. *B. melaninogenicus, B. intermedius, B. loescheii, B. denticola* and *B. corporis*, hydrolysed P-nitrophenyl-α-D-glucopyranoside and did not (except *B. intermedius*) produce indole. *B. loescheii* in addition hydrolysed three other enzyme substrates, while *B. melaninogenicus* hydrolysed two others, as shown in Table 3.

Under anaerobic conditions the N-acetyl-β-D-glucosaminidase activity of *B. gingivalis* was weak and variable and so was the α-fucosidase activity of *B. asaccharolyticus*. Tests conducted under aerobic conditions were more precise, consistent and convenient to perform.

These enzymatic activities were not demonstrable on Whatman number 1 filter paper saturated with enzyme substrate. The enzymatic activities were stable at pH range of 6.0–8.0 and the results were consistent after incubation in air for 12 or 24 h.

Incubation times required for the development of characteristic black pigmented colonies on the surface of blood media are shown in Table 4. Pure cultures of the asaccharolytic species did not grow on regular blood agar, but grew and developed characteristic black pigmented colonies on ETSA and anaerobic blood agar. Pure cultures of the saccharolytic species however grew on freshly prepared blood agar, ETSA and anaerobic blood agar. Development of pigmented colonies was enhanced and more rapid with increased concentration of blood in the media; pigmentation was produced more rapidly on agar supplemented with 9% sheep blood.

A scheme derived from the result of hydrolysis of enzyme substrates shown in Table 3 is presented in Fig. 1. The scheme was evaluated for the identification of the BPB species using clinical and reference strains of BPB. Accurate identification of these strains was usually possible within 4 h of aerobic incubation. The only short-coming observed was the somewhat inconsistent β-glucosidase activity of *B. loescheii* which may interfere with its separation from *B. melaninogenicus*.

Discussion

In this report, a simple scheme for the presumptive identification of BPB, derived from various enzymatic activities and haemagglutination of sheep erythrocytes, is presented.

The asaccharolytic BPB species, which are closely related phenotypically, were suc-

Table 4. Time of pigmentation by the human black pigmented *Bacteroides* species on different blood-containing media

Species	Pigment production range (days)				
	BA	ETSA	ABAP	ABAP1	ABAP2
B. gingivalis	0	4–7	4–7	3–5	2–4
B. endodontalis	0	4–8	4–8	4–6	3–5
B. asaccharolyticus	0	4–7	4–7	3–4	2–3
B. intermedius	3–6	3–5	3–5	2–4	2–3
B. melaninogenicus	3–6	3–5	3–5	2–4	2–3
B. denticola	3–6	3–5	3–5	2–4	2–3
B. corporis	3–6	3–5	3–5	2–4	2–3

BA = Blood agar; ETSA = enriched trypticase soy agar; ABAP1 = anaerobic blood agar with 7% blood; ABAP2 = anaerobic blood agar with 9% blood.

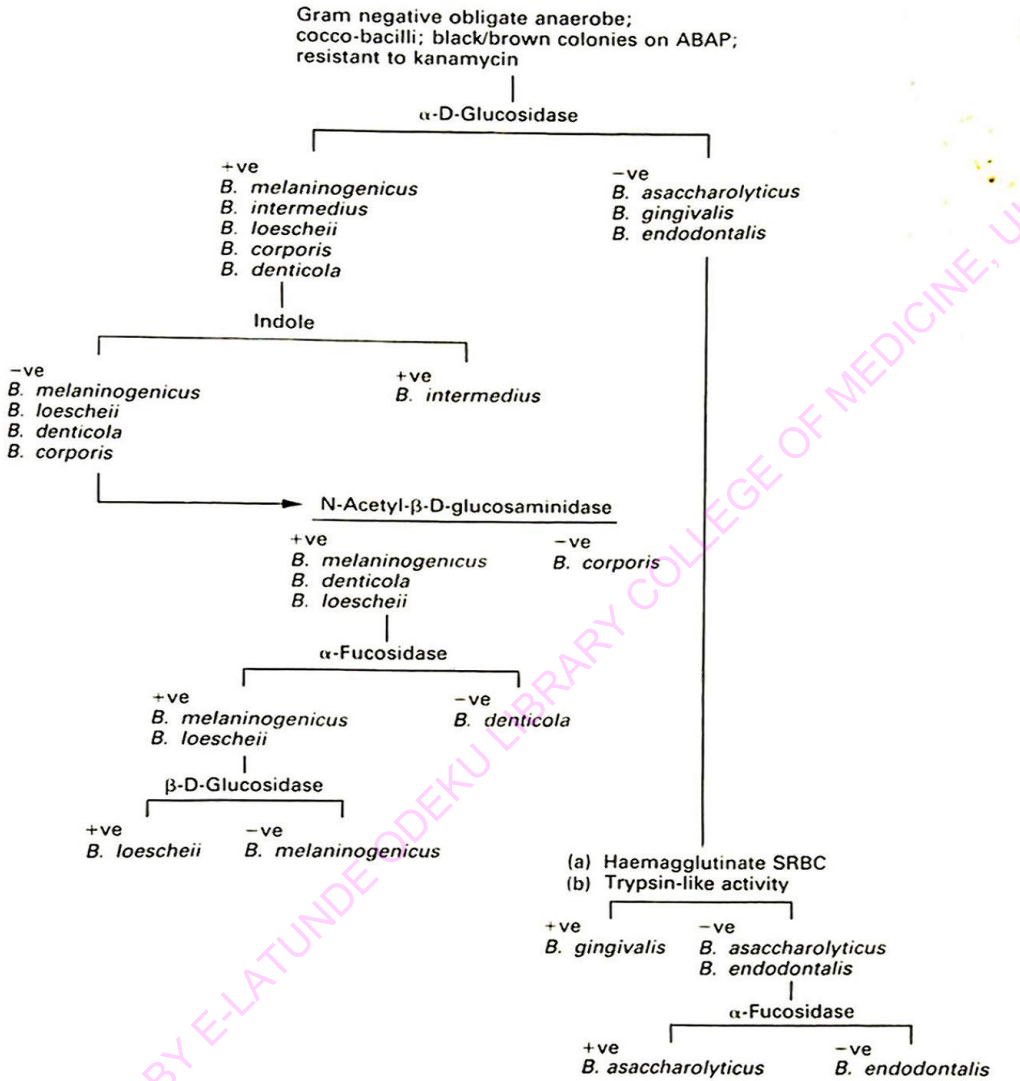

Fig. 1. Scheme for identification of human black pigmented *Bacteroides* species (ABAP = anaerobic blood agar plate; SRBC = sheep red blood cell).

cessfully differentiated by the differential ability to hydrolyse P-nitrophenyl-α-D-glucopyranoside, haemagglutination of sheep erythrocytes and production of trypsin-like activity. Normally, DNA studies and chromatographic analysis of metabolic end-products are required to differentiate the asaccharolytic species. Therefore, the ability to differentiate the asaccharolytic BPB by simple enzyme substrate hydrolysis is a significant finding, particularly in most laboratories of the Third World where funding and expertise are very scarce.

Differentiation of the saccharolytic species was readily achieved by their ability to hydrolyse α-D-glucopyranoside, α-fucopyranoside and N-acetyl-β-D-glucosaminide. Production of indole was useful in differentiating *B. corporis*

from *B. intermedius*, two species that were previously classified as one subspecies (*B. melaninogenicus* ss *intermedius*).

A problem occurring in the isolation of BPB is that they are usually present in mixed culture from clinical specimens and each culture may contain more than one *Bacteroides* species. For this reason it is inappropriate to pick black pigmented colonies at random from culture for the purpose of identification. It is more appropriate that pure cultures be obtained from a single colony for identification purposes.

The early formation of black pigmented colonies, which is an initial characteristic property, can be achieved by inoculating media with increased blood content. In our hands, addition of 9% sheep blood consistently enhanced and ensured early black pigmentation: this occurred usually about the second day of incubation on average. There is evidence that *B. gingivalis* and *B. asaccharolyticus* strains are inhibited by vancomycin [13,14], thus the use of selective media such as kanamycin/vancomycin blood agar [12], kanamycin/vancomycin laked blood agar [15] and anaerobe paromomycin/vancomycin blood agar [16] for isolating non-spore-forming Gram-negative anaerobes are unsatisfactory for primary or secondary cultivation of the BPB species.

The use of enzymatic activities for the rapid presumptive identification of anaerobes or capnophilic bacteria has been reported previously [17–20], and the use of chromogenic enzyme substrates has recently been introduced to a commercial identification scheme [20]. Chromogenic enzyme substrates are available commercially at reasonable cost and are quite stable during storage at 4°C. The nitrophenyl enzyme substrates used in this study were preferred to the others because of their sensitivity and ability to show hydrolysis within 4 h.

This scheme is practicable technically, reproducible, economical and reliable for rapid presumptive identification of the human BPB species within 4 h under aerobic conditions. Although it was found that the scheme could not be consistently used for differentiating *B. melaninogenicus* from *B. loescheii*, it identified successfully the clinically important strains, e.g. *B. intermedius, B. gingivalis* and *B. endodontalis*. In busy clinical laboratories this scheme could help to identify to species level more of the BPB isolated from clinical specimens. The speciation of the BPB should provide useful information about their causal association and possible role in many clinical infections.

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