

THE BLACK PIGMENTED BACTEROIDES SPECIES ISOLATED FROM NIGERIANS

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لقد تمت دراسة مدى انتشار الكائنات البكتيرية الدقيقة القمامية والمتوسطة والثوية في الأماكن السليمة، والأماكن المريضة من الفم. هذه الكائنات كانت تسمى في الماضي بالإصابات البكتيرية الملونة باللون الأسود. ولقد تم عزل البروفيتيلا المتوسطة بنسبة ٢٠٪ من الحالات القمامية في ٦٢٪ من الحفرة الفموية لدى ٥٠ شخصا سلبيا. ولم توجد على الإطلاق أي من الكائنات المسببة البروفيروماناس الثوية لدى هؤلاء الأشخاص الخمسون، علما بأن البروفيروماناس الثوية (٢٥٪) والمتوسطة (٤، ٥٣٪) هي من الأنواع المتواجدة قطعاً في الأفواه المريضة، ووجودها في حالات التقحح اللثوي المتعفن الحاد واضحا إحصائيا، وقد تم عزل هذه الأنواع في حالات خراج الأنسجة المحيطة بالأسنان وأيضا الكسور المتعفنة، والتقيحات في الأورام المكشوفة والتهابات العظام المتقيحة. هذا ولم تكن هناك أهمية إحصائية تربط هذه الأنواع بالتهابات الأنسجة المحيطة بالأسنان، ولكن لوحظ أنه كلما زاد عمر الشخص كلما زاد معدل عزل البكتريا المسببة بالثوية (جنجيفالس) ولوحظ أيضا أن بعض من هذه البكتريا مقاوم للمضاد الحيوي المسمى بالأمبسلين والتتراسيكلين والكيفالوثين والبتالكتانير المنتج والبالزما الحويصلية المحمولة التي ليس من الواضح طريقة عملها.

The prevalence of *Bacteroides meianinogenicus* and *B. intermedius* (now belonging to the *Prevotella* group) and *B. gingivalis* (*Porphyromonas* group) in healthy and diseased sites in the oral cavity was studied. The *P. melaninogenica*, *P. intermedia* and *P. gingivalis* were all formerly called black pigmented bacteroides (BPB). Out of the black pigmented bacteroides, *P. intermedia* and *P. melaninogenica* were isolated from 20% and 62% of fifty healthy oral cavities, respectively. No *Porphyromonas gingivalis* was isolated in this group of subjects. *P. gingivalis* (25%) and *P. intermedia* (53.4%) were positively associated with disease in the oral cavity. Their presence in acute necrotizing ulcerative gingivitis (ANUG) was statistically significant. They were also isolated from periodontitis, oral abscesses, infected fractures, infections superimposed on tumors, and suppurating osteomyelitis. There was a non-statistically significant association of these species with periodontitis. It was observed that the older the patient was, the more frequent the isolation rate of *P. gingivalis*. Some local isolates of *P. gingivalis* were resistant to ampicillin, tetracycline and cephalothin, produced betalactamase and carried cryptic plasmids whose functions were not clear.

Introduction

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The black pigmented *Bacteroides* species, now separated into *Prevotella* and *Porphyromonas* species, are part of the normal human oral flora' and are commonly isolated from both juvenile and adult periodontitis, necrotic pulp and odontogenic abscesses. There is evidence that *B. intermedius* (now called *P. intermedia*) and *B. gingivalis* (now *P. gingivalis*), in particular, possess virulence factors that are relevant to the pathogenesis of oral infections.^{9,10} Experimentally, *P. gingivalis* has

been shown to induce progression of periodontitis in laboratory animals.¹¹

Oral infections including the various clinical stages of periodontal diseases have been reported among Nigerian patients of all age groups.¹² However, there is no study so far which has established the prevalence or characteristics of black pigmented *Bacteroides* isolated from healthy or from oro-dental infections and diseases in Nigerians. Factors, such as the use of African chewing sticks for oral hygiene, diet, and exposure to antimicrobial agents, may cause variation in the composition and characteristics of the oral microflora in different populations.¹² Thus, it is necessary to establish the oral microbial flora (normal and pathogenic) in different populations in order to intelligently interpret the presence of some exotic microorganisms in clinical lesions.

This study was undertaken to investigate the prevalence and characteristics of some clinically important black pigmented *Bacteroides* species in specimens from healthy gingival crevices and orodental diseases among Nigerian populations.

Materials and Methods

Bacteria

Some bacterial strains were included for comparative characterization. These were *P. gingivalis* (ATCC 33277), *P. asaccharolyticus* (ATCC 25260), and *P. gingivalis* (CS 43; courtesy of Dr. S.S. Martins University of Maryland Dental School, Baltimore, MD). *P. gingivalis*, strain Barb's (courtesy of Dr. B.E. Laughon, John Hopkins Hospital, Baltimore, MD), and clinical strains of *P. gingivalis*, designated "Nig" isolated from oro-dental infections of Nigerians were also included in this study.

Subjects

Fifty adult Nigerians residing in Lagos with no oro-dental infections were recruited voluntarily for dental examination after verbal informed consent. One-hundred and three newly registered patients, presenting with a variety of oro-dental diseases, were selected from patients attending the dental clinic of the Lagos University Teaching Hospital (LUTH), Lagos, Nigeria between January 1984 and December 1986. All patients, who had used antibiotics for any form of dental treatment or

prophylaxis within the preceding six months, were excluded from the study. The relevant biodata, including age, sex, duration of disease, etc., were carefully recorded.

Media

The following solid and liquid media are used in the study: Anaerobic blood agar, plain blood agar, enriched chopped meat glucose broth (CMG) and BMK agar described by Holbrook *et al*¹³ containing no vancomycin, Amies transport medium (Oxoid) and one quarter strength Ringer's solution. All solid media plates were pre-reduced overnight before use at room temperature in an anaerobic jar with an atmosphere of 85% N₂, 10% H₂, 5% CO₂, or 90% H₂, 10% CO₂ as the case required.

Collections and Transportation of Specimens

Supra-gingival and sub-gingival materials were collected with sterile wooden toothpicks. When appropriate, materials were collected from abscesses or infected sites with serum coated swabs (Steriilin) or by aspiration using sterile syringes.

Materials collected with sterile toothpicks were transported in sterile vials containing 0.5 ml of pre-reduced one quarter strength Ringers solution with L-cysteine HCl (pH 7.2). Swabs were transported in Amies transport medium (Oxoid). Air was expelled from the syringes containing aspirated specimen materials and the needle bent to avoid inflow of air before transportation to the laboratory where all specimens were processed immediately.

Processing of Specimens

Plaque or pathologic materials transported in Ringer's solution were dispensed with sterile swabs (Steriilin) and inoculated directly onto anaerobic blood agar plates (ABAP), blood agar plates (BAP) and BMK agar plates. Materials were initially spread over a section of the plate and streaked out to obtain single colonies. Also, aspirated clinical materials or those collected with swabs were inoculated directly onto these media and streaked out to obtain single colonies. ABAP and BMK plates were immediately incubated anaerobically in the anaerobic jar at 37°C for 72 hours. For each specimen, two plain BAP were inoculated in air plus 10% CO₂ at 37°C. Each cultured specimen

was gram-stained and observed for Gram-negative coccobacilli and also inoculated into enriched cooked meat broth as a back-up for anaerobic cultures.

Representative black pigmented colonies on anaerobic agar plates were picked and subcultured onto fresh agar plates for purity. Isolates were tentatively identified by colonial appearance, gram-stain morphology and brick red fluorescence under ultraviolet light (Gallenkamp, England, 366 nm). Pure cultures were tested for indole and catalase production as well as for their ability to hemagglutinate sheep erythrocytes and ferment glucose. Resistance to colistin, kanamycin, and vancomycin was also noted. Isolates were then speciated according to the scheme in Table 1 culled from Wadsworth Anaerobic Bacteriology Manual.¹⁴

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Whole cell protein profiles of representative strains of *P. gingivalis* isolated from Nigerians were compared to those of the reference strains and other strains obtained from the USA to determine any geographic difference in clinical isolates. Preparation of strains for SDS-PAGE, gel preparation and running of samples were performed as previously described by Tanner *et al.*¹⁵

Antimicrobial Susceptibility Test

Antimicrobial susceptibility of test strains were determined by the disc diffusion method using the guidelines and interpretation of Barry.¹⁶ Briefly, a suspension of 72-hour culture was made in PBS reduced with dithiothreitol (DTT) and standardized to 0.5 MacFarland opacity. One milliliter of the suspension was flooded on anaerobic blood agar, drained and allowed to dry briefly before placing antibiotic discs on the surface of the agar. Four antibiotic discs were placed on each plate at equal distance and all manipulations were performed in an anaerobic chamber. The plates were then incubated under anaerobic conditions for 48 hours. Antibiotics tested were ampicillin (10 mcg), chloramphenicol (30 mcg), cephalothin (30 mcg), cefazolin (30 mcg), cefoxitin (30 mcg), cefotaxime (30 mcg), erythromycin (15 mcg), norfloxacin (10 mcg), novobiocin (5 mcg), metronidazole (4 mcg), rifampicillin (15 mcg), sulphamethoxazole/

trimethoprim (23.75/1.25 mcg), tetracycline (30 mcg), and tobramycin (10 mcg).

Biochemical Tests

Final strain identification was performed with procedures for biochemical test previously described by Dowell and Hawkins.¹⁷ Tests undertaken included the detection of indole, catalase, carbohydrate utilization, proteolysis, urease production, aesculin hydrolysis, hydrogen sulfide production and nitrate reduction (Table 1).

%Gas Liquid Chromatographic (GLC) Analysis of Metabolic End Products

Confirmation of strain identification was achieved by analysis of short chain volatile acids and non-volatile acid end products of amino acids, and sugar utilization were undertaken as previously described.^{18,9}

Hemagglutination Tests

Hemagglutination test was performed by the method of Slots and Genco,²⁰ to detect any differences in the hemagglutinating capabilities of the isolates from the two geographic areas.

Table 1. Simple identification scheme for black pigmented Bacteriodes isolates.

	<i>Porphyromonas gingivalis</i>	<i>Prevotella intermedia</i>	<i>Prevotella melaninogenica</i>
Source	Oral	Oral	Oral
Pigmentation	B/Br	B/Br	B/Br
Growth in:			
Anaerobic condition	+	+	+
Air	-	-	-
Candle jar	-	-	-
Cram Stain	GNCB	GNCB	GNCB
Indole	+	+	+
Catalase	-	-	-
Hemagglutination	+	-	-
Glucose utilization	-	+	+
Sensitivity to:			
Vancomycin	S	R	R
Kanamycin	R	R	R
Bacitracin	R	S	S
Metronidazole	S	S	S

Note: B/BR = Black/Brown
S = Sensitive
GNCB = Gram-negative
R = Resistant

Enzymatic Activity

Enzymatic activities for test strains were determined with the API- ZYM in IN-IDENT systems (Analytical Products, NY, USA). Each test was conducted and results were interpreted as recommended by the manufacturer. Trypsin-like activity was detected by procedures previously reported by Laughon *et al.*²¹

Beta-lactamase Detection

Beta-lactamase activity among representative isolates of *P. gingivalis* was detected by hydrolysis of the chromogenic cephalosporin (nigrocefim: BBL) following the procedures and interpretation recommended by the manufacturers.

Plasmid Studies

Test strains were screened for plasmid by using a modification of the alkaline lysis procedure for screening recombinant plasmid DNA.²¹ Extracted plasmid DNA were separated by electrophoresis on 0.7% agarose gel at 5 V/cm in trisborate buffer. Plasmid DNA were stained with ethidium bromide (1 µg/ml) and viewed under a violet transilluminator (265 nm).

Results

Plaque and supragingival specimens obtained from 31 (62%) and 10 (20%) of 50 healthy oral cavities yielded *P. melaninogenica* and *P. intermedia*, respectively. No *P. gingivalis* was isolated from these normal control specimens.

Of the 103 patients afflicted with oral diseases, *P. melaninogenicas* and *P. intermedia* were isolated from 47 (45.6%) and 55 (53.4%) of the clinical specimens, respectively. Noteworthy, *P. gingivalis* was isolated from 36 (34.9%) of these patients (Table 2). Statistically, this represents a 1.8 significant increased risk ($P \leq 0.0001$) of disease or infection in the oral cavities of Nigerians who were colonized by *P. gingivalis*. Also, a 1.35 significant risk of oro-dental diseases in patients colonized by *P. intermedia* was also recorded. However, there was no statistically significant association of *P. melaninogenica* with the oro-dental diseases or infections, when compared with the isolation rate in the control subjects ($p \geq 0.01$). The species was more common in the flora of healthy oral cavities than in the diseased cavities.

Table 2. Black pigmented Bacteroides (BPB) isolates from normal oral cavities and pathological sites.

Species	No. of BPB isolated at:	
	Normal site (N = 50)	Pathological site (N = 103)
<i>P. melaninogenica</i>	31	47
<i>P. intermedia</i>	10	55
<i>P. gingivalis</i>	0	36
<i>P. melaninogenica</i> / <i>P. intermedia</i>	10	27
<i>P. intermedia</i> / <i>P. gingivalis</i>	0	23
<i>P. melaninogenica</i> / <i>P. gingivalis</i>	0	12

The prevalence of *P. gingivalis* relative to *P. melaninogenica* and *P. intermedia* in clinical specimens obtained from patients with the various underlying oral pathologic conditions is shown in Table 3. Patients who suffered from acute necrotizing ulcerative gingivitis (ANUG) had a significant risk of colonization by *P. gingivalis* (RR4.28; $P \leq 0.01$), and *P. intermedia* (RR 7.24; $P \geq 0.02$).

There was a positive but non-significant relationship between colonization by *P. intermedia* (RR 1.1; $P \geq 0.01$) and *P. gingivalis* (RR 1.02; $P \geq 0.01$) and risk of periodontitis in Nigerians. *P. melaninogenica* showed no positive association. Except in periodontitis cases, the black pigmented bacteroides showed predilection for diseased sites in patients above 18 years. In patients with periodontitis, the prevalence of the three black pigmented *Bacteroides* in age-groups 0-18 and 18 was about the same.

Clinical specimens from 25 dento-alveolar abscesses yielded *P. melaninogenica* (56%), *P. intermedia* (72%) and *P. gingivalis* (32%).

The single case of suppurating mandibular osteomyelitis yielded only *P. gingivalis*, but other black pigmented bacteroides were isolated from a variety of other clinical conditions, namely infected fractures, infected dental cysts, infected tumors and facial cellulitis.

Characteristics of Nigerian Isolates of *P. gingivalis*

The representative isolates of *P. gingivalis* isolated from Nigerian patients were generally similar to those originally isolated from patients in the USA and exhibited similar cultural characteristics on anaerobic blood agar. An

Table 3. Isolation of black pigmented *Bacteroides* species from pathological sites.

Pathological Site	Number of sites	No. of species isolated per site						
		P.G	P.I	P.M	PGI	PGM	PMI	ALL
Dental caries	13	1	3	2	1	1	2	1
Pulpitis	9	2	3	6	1	2	2	1
Dentoalveolar abscess	25	8	18	14	4	1	8	1
Suppurating osteomyelitis	1	1	0	0	0	0	0	0
Infected fractures	4	2	2	1	0	0	1	0
Infected dental cyst	1	0	1	1	0	0	1	0
Infected non-dental cyst	1	0	0	0	0	0	0	0
Infected benign tumor	1	1	1	1	0	0	0	1
Infected malignant tumor	1	1	1	0	1	0	0	0
Facial cellulitis	2	0	1	2	0	0	1	0
Periodontitis	35	4	2	4	3	1	1	8
ANUG	10	7	9	7	6	4	7	4

P.G = *P. gingivalis* P.I = *P. intermedia* P.M = *P. melaninogenica* PGI = *P. gingivalis* & *P. intermedia*
 PGM = *P. gingivalis* & *P. melaninogenica* PMI = *P. intermedia* & *P. melaninogenica* ANUG = Acute necrotizing ulcerative gingivitis
 ALL = *P. gingivalis*, *P. intermedia* & *P. melaninogenica*

exception to this generalization was the "Barb" strain of *P. gingivalis*, which initially formed smaller translucent colonies that required longer incubation periods to form dark pigmented colonies. All the strains belonged to a relatively homologous group with respect to biochemical and enzymatic characteristics. They were indole positive, catalase negative, did not utilize carbohydrates and were proteolytic. These strains also produced trypsin-like enzyme, N-acetyl-B-D-glucosaminidase and alanine-amino-peptidase as determined by the API-ZYM in IN-DENT system. All of these strains, irrespective of source, exhibited the same hemagglutinating capabilities.

Chromatographic analysis showed that valeric, butyric, isovaleric and phenylacetic acids were the major end products of amino acid metabolism as with other standard reference strains. Minor end products detected were acetic, propionic, isobutyric and succinic acids. The Barb's strain of *P. gingivalis* produced quantitatively more phenylacetic acid than the other strains. Of all the *P. gingivalis* strains tested, only Barb's strain did not produce similar soluble whole cell protein bands on SDS-PAGE. An extra band was produced by Barb strain of *P. gingivalis* which was a high molecular weight protein band not present in the protein profiles of other strains.

The antibiogram for Nigerian isolates of *P. gingivalis* and other studies is shown in Table 4.

Most strains exhibited similar antimicrobial susceptibility to all the antibiotics. Some Nigerian strains were resistant to ampicillin, tetracycline and cephalothin. Four of the Nigerian strains produced beta-lactamase corresponding to those strains resistant to ampicillin and cephalothin. Two of these Nigerian strains resistant to ampicillin also carried cryptic plasmid bands very close to the chromosomal bands whose function was not clear.

Discussion

In this study, the characteristics of *P. gingivalis* isolated from Nigeria were not different from those of the strains isolated from other geographical areas. The only observable difference in the results was noticed with the sensitivity to vancomycin, protein profiles and enzymatic reactions produced by the "Barb" strain. Our investigation also confirmed the susceptibility of *P. gingivalis* to vancomycin as earlier reported by other workers.^{22,23} This apparently renders the addition of vancomycin to anaerobic media as selective media for isolation of *Bacteroides spp* inadvisable. Both *P. intermedia* and *P. melaninogenica* were isolated from the normal healthy gingival crevices of Nigerians in appreciable numbers. The nonisolation of *P. gingivalis* from healthy oral cavities agrees essentially with other previous reports from several geographic areas.²⁴⁻²⁸ Although *P.*

Table 4. Antimicrobial susceptibility patterns of *Porphyromonas gingivalis* strains studied.

Antibiotics	P. gingivalis strains									
	STD	CS	Barb	Nig 1	Nig 2	Nig 3	Nig 7	Nig 8	Nig 9	BA
Ampicillin	S	S	S	R	R	R	R	R	R	S
Cefazolin	S	S	S	S	S	S	S	S	S	S
Cefotaxime	S	S	S	S	S	S	S	S	S	S
Cefoxitin	S	S	S	S	S	S	S	S	S	S
Cephalotin	S	S	S	R	S	R	R	R	S	S
Chloramphenicol	S	S	S	S	S	S	S	S	S	S
Clindamycin	S	S	S	S	S	S	S	S	S	S
Erythromycin	S	S	S	S	S	S	S	S	S	S
Centamicin	R	R	R	R	R	R	R	R	R	R
Metronidazole	S	S	S	S	S	S	S	S	S	S
Norfloxacin	S	S	S	S	S	S	S	S	S	S
Sulphamethoxazole/ Trimethoprim	R	R	R	R	R	R	R	R	R	R
Tobramycin	R	R	R	R	R	R	R	R	R	R
Tetracycline	S	S	S	S	R	R	R	R	R	S
Rifampicin	S	S	S	S	S	S	S	S	S	S

S = Sensitive R = Resistant STD = P. gingivalis, ATCC 33277 CS = P. gingivalis strain, CS43 BA = P. asac- charolyticus

intermedia is considered to be a potentially pathogenic black pigmented *bacteroides*, BPB, it is also a prominent member of the normal microflora in non-inflamed oral cavities²¹⁻²⁴ which is in agreement with our present finding. *P. melaninogenica* was isolated in high numbers from healthy oral cavities of Nigerians and in relatively fewer number in pathological sites. It is widely acknowledged as part of the normal oral microflora in adults with no established serious pathogenic potential.^{9,29}

A large number of oral lesions were colonized by *P. gingivalis* and *P. intermedia*, and their presence at these sites showed a positive association with infection in the patients. Both species were also found to be common in ANUG lesions. Slots and Zambon²⁹ also found that *P. intermedia* constituted a significant part of the microflora in ANUG and isolated *P. gingivalis* from three of their four patients.

The consistent presence of these two bacterial species in periodontitis cases in this study, irrespective of age of the patients, suggested an association with the disease, even though not statistically significant. Earlier reports have shown that occurrence of these species in periodontitis normally decreases with decreasing age of

subjects.⁹ Other studies have indicated a significant association between *P. gingivalis* and periodontitis, and between *P. intermedia* and minimally inflamed periodontitis.^{6,26} A majority of the clinical specimens from abscesses and other suppurative infections yielded *P. gingivalis* and *P. intermedia* which was in agreement with some recent studies.^{20,30,31}

The only distinct dissimilarity between the Nigerian strain of *P. gingivalis* and the American strains studied was in respect to their antibiotic sensitivity patterns and perhaps the production of B-lactamase by the former. Some of the Nigerian strains also carried cryptic plasmids whose significance was not known. Unfortunately, we did not study enough number of strains from both countries to make a meaningful comparison of their phenotypic and genotypic characteristics. However, of significance is that the Nigerian strains were resistant to ampicillin and tetracycline, the two most widely used antibiotics in our community both for curative and prophylactic treatment of oral infections.

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