Selective Recovery of Oral *Capnocytophaga* spp. with Sheep Blood Agar Containing Bacitracin and Polymyxin B

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On the basis of in vitro susceptibility testing of antibiotics, dyes, and other antimicrobial agents, we developed and evaluated a medium, TBBP, for the selective isolation of oral *Capnocytophaga* spp. TBBP medium consists of 4% Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.), 5% sheep blood, 0.1% yeast extract, 50 µg of bacitracin per ml, and 100 µg of polymyxin B per ml. A total of 34 *Capnocytophaga* stock cultures grew well on TBBP medium. Except for some streptococcal strains, TBBP medium inhibited growth of all test stock culture isolates of common oral gram-positive and gram-negative species. In a clinical study of 15 deep periodontal pockets, TBBP medium demonstrated *Capnocytophaga* recoverability that was similar to or higher than that shown by a nonselective blood agar medium. Typical *Capnocytophaga* colonial morphology enabled us to readily distinguish this organism from the few other bacteria which could grow on TBBP medium.

Capnocytophaga organisms are capnophilic gram-negative long rods which glide or swarm on certain agar media (14, 18, 23). Their primary ecological niche is dental plaque.

Capnocytophaga species have been implicated as important agents in localized juvenile periodontitis (13, 15), periodontitis associated with insulin-dependent diabetes mellitus (P. A. Mashimo, Y. Yamamoto, J. Slots, B. H. Park, and R. J. Genco, J. Periodontol., in press), and Papillon-Lefèvre syndrome (11). These periodontal diseases are associated with defective functions of polymorphonuclear leukocytes (20). In the more common forms of periodontitis, *Capnocytophaga* spp. may have little or no etiologic role (10).

Capnocytophaga spp. can be important pathogens in cytopenic and immunocompromised hosts. There have been reports of seven patients with acute leukemia (5, 6, 22), one patient with adenocarcinoma (6), and one patient with Hodgkin's disease (1) who developed *Capnocytophaga* sepsis. These patients demonstrated marked signs of periodontal disease, and the *Capnocytophaga* entrance was probably the periodontal tissues.

Although the evidence is still sparse, the available information suggests that in immunodeficient patients, *Capnocytophaga* spp. can be serious pathogens that cause dental as well as systemic infections. Because proper therapy for *Capnocytophaga* infections may depend on the isolation and identification of the organism, our

aim was to develop a medium for selective *Capnocytophaga* recovery.

MATERIAL AND METHODS

Antimicrobial agents. Antibiotics, dyes, and other antimicrobial substances used were as follows. Rifampin, ampicillin, penicillin G potassium salt, tetracycline hydrochloride, erythromycin, streptomycin sulfate, gentamicin sulfate, neomycin sulfate, bacitracin, polymyxin B sulfate, sodium fluoride, and neutral red were from Sigma Chemical Co., St. Louis, Mo. Cadmium sulfate, safranin O, Evans blue, toluidine blue O, bromocresol green, methylene blue, malachite green, and crystal violet were from Fisher Scientific Co., Fair Lawn, N.J. Minocycline hydrochloride was from Lederle Laboratories, Pearl River, N.Y. Doxycycline hyclate and disodium carbenicillin were from Pfizer Inc., New York. Chloramphenicol was from Parke, Davis & Co., Morris Plains, N.J. Sodium cefoxitin was from Merck, Sharp & Dohme Research Laboratories, West Point, Pa. Sodium oxacillin was from Bristol Laboratories, Syracuse, N.Y. Ruthenium red, Alcian blue, diazo blue B, and lissamine green were from K & K Laboratories, Inc., Plainview, N.Y. Azocarmine B and eriochrome blue SE were from Allied Chemical & Dye Co., New York. Superchrome blue B extra was from Nutritional Biochemicals Corp., Cleveland, Ohio. Bromocresol purple was from BBL Microbiology Systems, Cockeysville, Md.

Bacterial strains. The following bacteria, obtained from the American Type Culture collection (ATCC), Rockville, Md., and from our own laboratory (State University of New York at Buffalo [SUNYab]), were tested for susceptibility to antimicrobial agents: *Capnocytophaga* spp. 34 SUNYab strains; *Actinobacillus actinomycescomitans*, 16 SUNYab strains, 1 Y4

strain from S. S. Socransky, Boston, Mass., and ATCC 29522, ATCC 29523, and ATCC 29524; *Fusobacterium nucleatum*, 19 SUNYaB strains and ATCC 25586; *Bacteroides asaccharolyticus*, 4 SUNYaB strains and ATCC 27067; *B. gingivalis*, 1 SUNYaB strain; *B. melaninogenicus* subsp. *intermedius*, 4 SUNYaB strains; *B. melaninogenicus* subsp. *melaninogenicus*, 1 SUNYaB strain and ATCC 15930; facultative *Actinomyces* species, 14 SUNYaB strains; and *Streptococcus* species, 15 SUNYaB strains. The SUNYaB strains, with the exception of the nonoral *B. asaccharolyticus* strains, originated from supragingival and subgingival dental plaque.

Cultures were either maintained by weekly transfer on blood agar plates or frozen at -80°C .

Growth in the presence of inhibitory agents. Preliminary experiments revealed that *Capnocytophaga* spp. grew poorly on Wilkins Chalgren agar (Difco Laboratories, Detroit, Mich.), a medium commonly used in susceptibility assays. Susceptibility assays were therefore carried out with TB medium, which contained the following: 40 g of Trypticase soy agar (BBL Microbiology Systems), 1 g of yeast extract (Difco Laboratories), 2 g of glucose, 50 ml of defibrinated sheep blood, and 960 ml of distilled water. Before the sheep blood was added, the pH of the medium was adjusted to 7.2 and the medium was autoclaved for 15 min at 121°C . After the medium was cooled at 50°C , blood and inhibitory agents were added to give the appropriate final concentrations. Each inhibitory agent was sterilized by filtration. The plates were stored aerobically at 5°C and used within 7 days of preparation.

Bacterial inocula were prepared from 48-h anaerobic cultures of the organisms in enriched brain heart infusion broth (BBL Microbiology Systems). The cultures were diluted to a turbidity of one McFarland no. 1 barium sulfate standard, dispensed with a Steers replicator (Melrose Machine Shop, Woodlyn, Pa.) onto the test plates, and incubated for 48 h at 37°C in a Coy anaerobic chamber (Coy Manufacturing Co., Ann Arbor, Mich.) containing 85% N_2 , 10% H_2 , and 5% CO_2 . The results were recorded as positive if more than three discrete colonies or a distinctly visible haze developed.

Clinical specimens. After in vitro susceptibility data were collected, a selective medium of TB supplemented with 50 μg of bacitracin per ml and 100 μg of polymyxin B per ml (TBBP medium) was made up. To evaluate the efficacy of the TBBP medium in the recovery of *Capnocytophaga* spp. from oral specimens, five patients, 16 to 20 years old, with severe periodontitis and insulin-dependent diabetes mellitus were studied. Samples from three deep periodontal pockets in each patient were obtained with paper points as previously described (17). Each sample was transferred to 9 ml of anaerobic Ringer solution, the bacteria were dispersed with a Vortex mixer at the maximal setting for 60 s, and the bacterial suspension was serially diluted in 10-fold steps in anaerobic Ringer solution. With a bent glass rod, we plated 0.1-ml portions of appropriate dilutions on TB and TBBP media. The plates were incubated at 37°C for 3 days in a Coy anaerobic chamber, and the total number of colonies and the number of surface-translocating, wet, flat, medium-to-large colonies suggestive of *Capnocytophaga* spp. were enumerated. Representative isolates suspected of being *Capnocytophaga* spp. were

TABLE 1. Susceptibility of 34 *Capnocytophaga* strains to antibiotics and other antimicrobial agents^a

Agent	MIC range ^b	MIC median ^c
Ampicillin	0.012–0.19	0.048
Bacitracin	100–>200	>200
Carbenicillin	0.39–3.13	0.78
Chloramphenicol	1.56–12.5	3.13
Cefoxitin	0.39–6.25	1.56
Doxycycline	0.096–0.78	0.19
Erythromycin	0.19–3.13	0.59
Gentamicin	50–>200	100
Minocycline	0.048–0.39	0.096
Neomycin	100–>200	>200
Oxacillin	3.13–25	6.25
Penicillin	0.024–0.39	0.048
Polymyxin B	>200	>200
Rifampin	0.006–0.048	0.012
Streptomycin	25–200	100
Tetracycline	0.096–1.56	0.39
Cadmium sulfate	1.56–25	5.70
Sodium fluoride	25–>200	200

^a Concentrations of antimicrobial agents tested: doubling dilutions from 0.003 to 200 $\mu\text{g}/\text{ml}$.

^b Minimal inhibitory concentration (MIC) is expressed in micrograms per milliliter.

^c Lowest antimicrobial concentration capable of inhibiting 50% of the strains tested (expressed in micrograms per milliliter).

subcultured and confirmed as *Capnocytophaga* spp. by the appropriate morphological and biochemical identification criteria (14, 18).

RESULTS

Susceptibility data for the *Capnocytophaga* spp. are given in Table 1. *Capnocytophaga* spp. were not inhibited by any test concentration of polymyxin B, and only 1 of the 34 test strains was inhibited by 100 μg of bacitracin per ml. *Capnocytophaga* spp. were highly susceptible to antibiotics of the penicillin and tetracycline groups. Cadmium sulfate, which has been utilized as an inhibitory agent in selective media for oral organisms (2, 9), inhibited *Capnocytophaga* growth at relatively low concentrations.

Capnocytophaga strains were susceptible to some dyes, especially malachite green and crystal violet (Table 2). The strains were not inhibited by Alcian blue, diazo blue B, and lissamine green at 500 $\mu\text{g}/\text{ml}$. However, these three dyes did not inhibit strains of several other oral species tested (Mashimo, unpublished data) and therefore were not considered valuable agents for a *Capnocytophaga* selective medium.

Table 3 lists the effects of bacitracin and polymyxin B on representative strains of major oral bacterial species. None of the *Capnocytophaga* strains was inhibited by a combination of 50 μg of bacitracin per ml and 100 μg of poly-

TABLE 2. Susceptibility of 34 *Capnocytophaga* strains to dyes^a

Dye	MIC range ^b	MIC median ^c
Alcian blue	>500	>500
Azocarmine B	50->500	>500
Bromcresol green	50->500	>500
Bromcresol purple	100->500	>500
Crystal violet	<1	<1
Diazo blue B	>500	>500
Eriochrome blue SE	50->500	500
Evans blue	5-500	75
Lissamine green	>500	>500
Malachite green	<1	<1
Methylene blue	50-500	500
Neutral red	5->500	100
Ruthenium red	50-500	500
Safranin O	1-500	50
Suprachrome blue B	1-100	50
Toluidine blue O	5->500	500

^a Concentrations of dyes tested: 1, 5, 50, 100, and 500 µg/ml.

^b Minimal inhibitory concentration (MIC) is expressed in micrograms per milliliter.

^c The lowest dye concentration capable of inhibiting 50% of the strains tested (expressed in micrograms per milliliter).

myxin B per ml. One-third of the streptococcal strains grew in the presence of these bacitracin and polymyxin B concentrations. In contrast, all remaining test strains were entirely inhibited by this combination of bacitracin and polymyxin B.

A total of 15 specimens from advanced periodontitis lesions were obtained. Table 4 shows the total viable counts and the number of *Capnocytophaga* colonies recovered on TB and TBBP media. *Capnocytophaga* spp. were present in each sample and constituted an average of 13% of the cultivable microorganisms on TB medium. On TBBP medium, however, *Capnocytophaga* spp. constituted 80% of the total isolates, meaning that only 20% of the colonies were contaminating organisms, mainly *Streptococcus* species and occasionally *Veillonella* species and a few other unclassified gram-negative bacteria.

The colonies of the contaminants differed from those of *Capnocytophaga* spp. by being smaller and showing no tendency to swarm on the agar surface. For 7 of the 15 clinical specimens, the *Capnocytophaga* colony counts on TBBP medium were >25% higher than the counts on TB medium. The average recovery of *Capnocytophaga* spp. on TBBP medium exceeded that on TB medium (median value, 19%).

DISCUSSION

Although several investigators have examined the in vitro susceptibility of *Capnocytophaga* spp. to dyes (18) and to antibiotics and other antimicrobial agents (4, 12, 19, 21), no previous study has attempted to devise and evaluate a selective medium for these organisms.

A high in vitro *Capnocytophaga* resistance to bacitracin and polymyxin B is of interest to selective medium development. In oral microbiology, bacitracin is included in several selective media (7, 8, 16) because of its ability to inhibit a large portion of the oral microbiota. Polymyxin B has been utilized to suppress contaminating pseudomonads on mitis salivarius agar (3). Our results showed that polymyxin B at concentrations of 50 to 100 µg/ml can also be used to inhibit some bacitracin-resistant, gram-negative bacteria indigenous to the oral cavity.

The performance of TBBP medium was evaluated in a clinical trial. We found that for 7 of 15 periodontal pocket specimens, the numbers of *Capnocytophaga* colonies on TBBP medium exceeded those on nonselective blood agar medium by more than 25%. These data suggest that some oral organisms growing on the nonselective medium inhibit the growth of *Capnocytophaga* spp. Indeed, oral streptococci and other organisms have demonstrated high in vitro activity against *Capnocytophaga* spp. (Y. Yamamoto, P. A. Mashimo, H. Reynolds, and R. J. Genco, Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, D41, p. 50; L. H. Satin and V. L. Sutter, Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, D42, p. 50). A major advantage of TBBP

TABLE 3. Effects of bacitracin and polymyxin B on oral bacteria

Bacterium (no. of strains)	Total no. of inhibited strains/total no. of strains tested				
	Bacitracin		Polymyxin B		Bacitracin (50 µg/ml) + polymyxin B (100 µg/ml)
	25 µg/ml	50 µg/ml	50 µg/ml	100 µg/ml	
<i>Capnocytophaga</i> spp. (34)	0/34	0/34	0/34	0/34	0/34
<i>A. actinomycetemcomitans</i> (20)	0/20	0/20	20/20	20/20	20/20
<i>F. nucleatum</i> (20)	0/20	1/20	20/20	20/20	20/20
<i>B. asaccharolyticus</i> (5)	4/5	5/5	0/5	0/5	5/5
<i>B. gingivalis</i> (1)	1/1	1/1	0/1	0/1	1/1
<i>B. melaninogenicus</i> subsp. <i>intermedius</i> (4)	4/4	4/4	4/4	4/4	4/4
<i>B. melaninogenicus</i> subsp. <i>melaninogenicus</i> (2)	1/2	2/2	0/2	0/2	2/2
Facultative <i>Actinomyces</i> spp. (14)	14/14	14/14	0/14	0/14	14/14
<i>Streptococcus</i> spp. (15)	3/15	9/15	0/15	1/15	10/15

TABLE 4. Recovery of *Capnocytophaga* spp. from periodontal pocket samples on nonselective TB medium and on TBBP medium

Patient no.	Periodontal site	Total colony counts (×10 ⁶)		Capnocytophaga spp. on indicated medium				% Increase in Capnocytophaga recovery on TBBP ^a
				Total no. (×10 ⁶)		% of total colony counts		
		TB	TBBP	TB	TBBP	TB ^b	TBBP ^c	
1	1	772	123	79	85	10	69	8
	2	326	48	30	39	9	81	30
	3	245	55	30	48	12	89	63
2	1	536	145	95	102	18	70	7
	2	626	117	109	94	17	80	−13
	3	415	61	41	54	10	89	31
3	1	181	41	19	29	10	70	53
	2	388	74	61	66	16	89	8
	3	750	176	142	150	19	85	6
4	1	278	52	31	39	11	75	26
	2	233	49	33	42	14	86	27
	3	458	65	20	55	9	85	175
5	1	650	130	112	104	13	80	−7
	2	721	129	91	101	13	78	11
	3	692	111	88	90	13	81	2

^a Compared with recovery on TB. TBBP grew statistically significantly more *Capnocytophaga* spp. than did TB ($0.001 < P < 0.005$), as determined by the Wilcoxon paired-sample test (24). Mean, 28%; median, 19%.

^b Mean, 13%; median, 13%.

^c Mean, 80%; median 81%.

medium is that it apparently suppresses inhibitory organisms and thereby maximizes *Capnocytophaga* recovery.

In summary, this report describes a selective medium for *Capnocytophaga* spp. which can easily be made up in a clinical microbiology laboratory. The medium has a high recovery efficiency for *Capnocytophaga* spp., and the few contaminating organisms can be readily distinguished from *Capnocytophaga* spp. on the basis of colonial morphology. TBBP medium should be useful in the bacteriological diagnosis of oral and nonoral *Capnocytophaga* infections.

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