Cultural and Biochemical Characteristics of Clinical Isolates of Unusual Colistin-Resistant *Pseudomonads*

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Biochemical characteristics and antibiotic susceptibilities of 12 strains of colistin-resistant pseudomonads isolated from clinical specimens are reported. The isolates were short, oxidase-positive, nonfluorescing, gram-negative rods that failed to grow on salmonella-shigella or cetrимide agars, to decarboxylate amino acids, and to reduce nitrates. Most strains peptonized litmus milk and grew at 42°C. Glucose, lactose, maltose, xylose, and fructose were slowly oxidized, whereas sucrose was not. Two homogeneous species were found and tentatively listed as *Pseudomonas* sp. 1 and *Pseudomonas* sp. 2, and these were differentiated by gelatin and starch hydrolysis, oxidation of mannitol, and alkalization of allantoin. The two species were shown to differ from the Center for Disease Control Va group biotype CDC Va-1 in both biochemical characteristics and susceptibility to the aminoglycosides.

The classification of nonfermentative gram-negative bacteria has been incomplete as evidenced by the still large number of organisms without species rank (13, 16). These bacteria are designated by number and letter and occupy an apparent taxonomic position close to similar, well-defined strains. Indicative of such organisms is the currently recognized Center for Disease Control (CDC) Va group, previously regarded as group IVd (4). Two well-delineated biotypes within the Va group have been reported; one of these (CDV Va-2) is seemingly identical to the newly described *Pseudomonas pickettii* (11, 12). However, the variation in ability to produce acid from most carbohydrates suggests that these organisms are heterogeneous and that the group consists of numerous biotypes (13).

During a 3-year period, 12 strains of oxidase-positive, colistin-resistant, gram-negative, nonfermenting bacteria, bearing a reluctance to attack carbohydrates rapidly and falling into two separate categories of *Pseudomonas*, have been recovered from a variety of clinical specimens. The first culture isolated was reminiscent of a lysine-negative *Pseudomonas cepacia* strain, although subsequent examinations did not bear this out.

This report presents the findings of tests to determine a wide variety of biochemical and nutritional properties and of tests for susceptibility to antimicrobial agents obtained with these isolates. Additional data are presented which compare the resultant characteristics with

those obtained with members of currently recognized taxa.

MATERIALS AND METHODS

Organisms tested. The 12 bacterial isolates were recovered from clinical materials submitted for examination to the Microbiology Laboratory, Madigan Army Medical Center. The five strains listed as *Pseudomonas* sp. 1 were isolated from eye, gall bladder, skin, blood, and cervical cultures. The seven isolates of *Pseudomonas* sp. 2 were recovered from rectal and cervical cultures (two strains each) and from urethral, nasal, and hydrocele cultures. No attempt was made to assign an etiological role to the isolates. Cervical and urethral isolates were recovered on modified Thayer-Martin medium during isolation attempts for the gonococci; the remainder of the isolates were recovered on 5% defibrinated sheep blood and MacConkey agars. Blood and modified Thayer-Martin media were incubated under 5 to 7% CO₂, and MacConkey agar was incubated under ambient air at 35°C. Colonies were visible on all media after overnight incubation at 35°C.

Biochemical tests. The media and methods used for identification have been described previously (9). Acid production from carbohydrates was determined using King semisolid oxidation/fermentation media containing phenol red indicator, pH 7.3 (9, 16). The test for urease was on Christensen medium (9). Alkalization of amides and salts (9) was tested on Simmons agar base (Difco Laboratories, Detroit, Mich.) at a 0.5% concentration, except for acetamide, which was tested at a 1% concentration. Growth at 42°C was on Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) slants (8). Identification procedures were initiated by subculturing to Trypticase soy broth colonies taken from MacConkey agar or modified Thayer-Martin medium. One drop or loopful of an 18-

to 24-h broth suspension then was used to inoculate the test media. The tests were incubated at 35°C, with additional tubes of pseudomonas F and P agars (Difco), potassium gluconate, and nutrient gelatin incubated at 25°C.

Antimicrobial susceptibility tests. All strains were tested for susceptibility to 14 antibiotics according to standardized procedures (7) using the disk diffusion test on Mueller-Hinton agar. Minimal inhibitory concentrations were determined using commercially prepared antibiotic trays (Prepared Media Laboratories, Tualatin, Oreg.). The trays were examined after overnight incubation at 35°C and permitted to incubate for an additional 6 h at room temperature before the control wells contained satisfactory growth for reading purposes.

RESULTS

After overnight incubation on sheep blood agar, all isolates grew as tiny, grayish-white, convex colonies with entire edges, approximately 0.5 mm in diameter. The colony size increased to 1.0 mm with continued incubation. Growth was not enhanced by CO₂, nor was hemolysis evident on sheep blood agar. On MacConkey agar the isolates characteristically appeared as tiny non-lactose-fermenting colonies after 24 h, with an increase in size after continued incubation. All cultures grew well on modified Thayer-Martin medium, and their appearance was remarkably similar to that of the gonococci.

Biochemical characteristics. The results of the biochemical tests are shown in Table 1. The organisms were characterized as short, oxidase-positive, nonfluorescing, gram-negative rods that failed to grow on salmonella-shigella or cetrimide agars, to decarboxylate amino acids or to attack nitrates. Most strains peptonized litmus milk and grew at 42°C. Glucose, lactose, maltose, xylose, and fructose were oxidized, whereas sucrose was not. Motility varied according to strain. Flagella-stained preparations revealed few flagellated cells; these showed monopolar flagella. *Pseudomonas* sp. 1 differed from *Pseudomonas* sp. 2 in that the latter liquefied gelatin, hydrolyzed starch, and actively oxidized mannitol, whereas the former failed to do so. Significantly, all strains of *Pseudomonas* sp. 2 developed a slight yellow pigment on Mueller-Hinton agar with extended incubation. All strains of *Pseudomonas* sp. 1 alkalinized allantoin, whereas those of *Pseudomonas* sp. 2 did not.

Several features distinguished the strains of the two species from those of CDC Va-1. The major differences were that Va-1 isolates reduced nitrates and alkalinized tartrate, but failed to peptonize litmus milk and were less consistent in oxidation of carbohydrates.

The prominent features of *Pseudomonas* sp. 1 and 2 are shown in Table 2. Alkalinization of allantoin and tartrate and acidification of mannitol were distinguishing features of these Va-like organisms.

Antimicrobial susceptibility. The susceptibility patterns of the 12 strains are shown in Tables 3 and 4. Comparative data for other colistin-resistant organisms are also shown. All isolates were uniformly resistant to ampicillin, carbenicillin, gentamicin, streptomycin, tobramycin, and amikacin and uniformly susceptible to tetracycline and sulfamethoxazole. Although *Pseudomonas* sp. 2 organisms were intermediate in susceptibility to cephalothin, chloramphenicol, and kanamycin when tested by the disk diffusion test, they were resistant when tested by the broth dilution test (Table 4). *Pseudomonas* sp. 1 organisms were resistant to cephalothin, chloramphenicol, and kanamycin when tested by both techniques. Strains of Va-1 were variable in their response to the antimicrobial agents. Of interest was the susceptibility of the Va-1 isolates to the aminoglycosides gentamicin, tobramycin, and amikacin in contrast to the two unknown species. Except for the aminoglycosides and tetracycline, the correlation in findings with the disk diffusion and broth dilution tests was not close.

DISCUSSION

The organisms designated as group Va constitute a heterogeneous collection of oxidase-positive, monopolar, gram-negative rods which fail to fluoresce, to grow on cetrimide, or to attack lysine or arginine, but which oxidize a variety of carbohydrates (13). Two distinct varieties are currently recognized (13), and one of these, CDC Va-2, has been given species rank as *P. pickettii* (11, 12). Recently, a denitrifying saprophyte recovered from soil and bearing a biochemical similarity to *P. pickettii* has been described (2). Isolates of the Va group and related organisms are rarely reported in the literature, and the paucity of detailed descriptions of these organisms contributes to the confused taxonomy. The failure to recognize the Va group may result from the poor growth on selective media after 24 h of incubation, from overgrowth by accompanying organisms on blood agar, from the similarity of reactions to "atypical" organisms of currently recognized taxa, or simply from the lack of careful consideration of unusual strains when they are encountered in clinical specimens.

The group IVd is not currently recognized, although these organisms are accepted as synonymous with the broad group Va. The taxon IVd (4) seems to differ from Va only in the

TABLE 1. Biochemical characteristics of colistin-resistant *Pseudomonas* species^a

Test or substrate ^b	<i>Pseudomonas</i> sp. 1 (5 strains)		<i>Pseudomonas</i> sp. 2 (7 strains)		CDC Va-1 (5 strains)	
	+(+)	%	+(+)	%	+(+)	%
Motility (slide)	5	100	6	86	3	60
Motility (tube)	5	100	5	71	2 (1)	60
Oxidase	5	100	7	100	5	100
Urease	3 (2)	100	5 (2)	100	4 (1)	100
Phenylalanine	0	0	0 (2) ^w	29	0	0
Nitrite produced	0	0	0	0	2 (1)	60
Denitrification	0	0	0	0	2	40
Gelatinase (35°C)	0	0	0 (4)	57	0 (2)	40
Gluconate oxidation	0	0	0	0	0	0
Cetrimide tolerance	0	0	0	0	0	0
Esculin hydrolysis	0	0	0	0	0	0
Starch hydrolysis	0	0	5	71	1	20
Deoxyribonuclease	0	0	0	0	0	0
Lecithinase	0	0	0	0	0	0
ONPG ^c (β -D-galactosidase)	1 ^w	20	0	0	1	20
Pigment	0	0	7	100	1	20
Salmonella-shigella agar	0	0	0	0	0	0
MacConkey agar	5	100	7	100	5	100
Hemolysis	0	0	0	0	0	0
Lysine decarboxylase	0	0	0	0	0	0
Arginine dihydrolase	0	0	0 (2) ^w	29	0	0
Ornithine decarboxylase	0	0	0	0	0	0
Growth in 6.5% NaCl ^d	0	0	0	0	0	0
Growth at 42°C ^d	2	50	6	86	1	33
Litmus milk peptonized	0 (5)	100	0 (5)	71	0	0
Oxidation/fermentation						
Glucose	5	100	7	100	4 (1)	100
Lactose	4 (1)	100	4 (3)	100	0 (4)	80
Sucrose	0	0	0	0	0 (1)	20
Maltose	4 (1)	100	3 (2)	71	1 (2)	60
Mannitol	0	0	6 (1)	100	1 (1)	40
Xylose	5	100	7	100	3 (2)	100
Fructose	3 (2)	100	7	100	2 (2)	80
Amides and salts						
Acetamide	0	0	0	0	0	0
Allantoin	5	100	0	0	5	100
Asparagine ^d	5	100	7	100	3	100
Butyramide	0 (5)	100	0 (3)	43	1 (3)	80
Citrate	5	100	7	100	5	100
Formamide	5	100	7	100	4 (1)	100
Hippurate ^d	5	100	7	100	3	100
Malonate	5	100	7	100	3 (2)	100
Nicotinamide	0	0	0	0	0	0
Propionate	4 (1)	100	0 (4)	57	1 (2)	60
Tartrate	0	0	0	0	4 (1)	100

^a +, Number positive within 2 days; (+), number positive within 3 to 7 days; %, percent positive after 7 days; W, weak reaction.

^b All strains were negative for the following tests: H₂S, pyocyanine, and fluorescein production.

^c ONPG, o-Nitrophenyl- β -D-galactopyranoside.

^d Most but not all strains were tested against the substrate.

ability of the organisms in the latter group to denitrify (13). The IVd organisms have been described as strongly urease positive, but failing to reduce nitrates or oxidize lactose and sucrose (5). On the other hand, they have been reported to acidify sucrose, lactose, xylose, and maltose in 5 days, and to reduce nitrate (3). In most

cases, however, the characteristics of the IVd group have been poorly described (6). The organisms have been implicated in respiratory disease (5) and fatal endocarditis (3) and have been recovered from inhalation equipment (6). Va-1 organisms have been responsible only for one case of serious human infection, acute meningitis

TABLE 2. Comparison of *Pseudomonas* spp. 1 and 2 to other biochemically similar nonfermentative bacteria^a

Test or substrate	<i>Pseudomonas</i> sp. 1 (5)	<i>Pseudomonas</i> sp. 2 (7)	CDC Va-1 (5)	<i>P. cepacia</i> (8)	CDC Vd-1 (5)	<i>Achromobacter</i> sp. 1 (8) ^b	<i>Achromobacter</i> sp. 2 (3) ^b	<i>P. paucimobilis</i> (19)	CDC IIK-2 (9)
Gelatin liquefaction	0	57	40	75	0	0	0	5	0
Pigment (slight yellow)	0	100	20	0	0	0	0	100	67
Nitrite production	0	0	60	63	60	50	33	0	11
N ₂ gas production	0	0	40	0	100	13	100	0	0
Urease production	100	100	100	88	100	100	67	0	100
Esculin hydrolysis	0	0	0	75	100	100	0	100	100
ONPG ^c (β -D-galactosidase)	20	0	20	100	0	100	0	74	89
Litmus milk peptonized	100	71	0	88	0	88	0	5	0
Sucrose	0	0	20	75	0	100	0	95	100
Maltose	100	71	60	100	0	100	0	100	100
Mannitol	0	100	40	75	0	88	0	0	0
Allantoin	100	0	100	0	100	100	100	0	100
Tartrate	0	0	100	75	20	0	0	14	0
Flagellar arrangement	1	1	1	>2	P ^d	P	P	— ^e	—

^a Numbers within parentheses indicate the number of strains tested. Results expressed as percent positive after 7 days of incubation.

^b See reference 9.

^c ONPG, o-Nitrophenyl- β -D-galactopyranoside.

^d P, Peritrichous.

^e —, No flagella.

TABLE 3. Susceptibility of colistin-resistant, oxidative, nonfermentative bacteria to 14 antibiotics^a

Antibiotic	<i>Pseudomonas</i> sp. 1 (5)	<i>Pseudomonas</i> sp. 2 (7)	CDC Va-1 (4)	<i>P. pickettii</i> (2)	<i>P. cepacia</i> (8)	<i>P. paucimobilis</i> (17)	CDC IIK-2 (7)
Ampicillin	0 ^b	0	0	0	0	82	43
Carbenicillin	0	0	50	100	13	94	100
Cephalothin	0	43	75	100	0	29	14
Chloramphenicol	0	86	75	100	100	94	100
Kanamycin	20	71	100	50	63	71	0
Gentamicin	0	0	100	0	25	94	29
Tetracycline	100	100	100	100	0	100	100
Streptomycin	0	0	50	0	0	0	29
Sulfamethoxazole	100	100	100	100	100	75	80
Nitrofurantoin	0	0	0	0	0	29	60
Tobramycin	0	29	100	0	0	100	0
Amikacin	0	0	100	0	0	100	0
Nalidixic acid	— ^c	100	100	100	100	29	100
Trimethoprim/sulfamethoxazole	—	—	100	100	100	100	100

^a Colistin resistance determined by agar disk diffusion. Numbers within parentheses indicate the number of strains tested.

^b Results expressed as percent susceptible. Percentages include susceptible and intermediate results.

^c —, No data available.

(1).

Von Graevenitz (14) reported that IVd organisms were observed as contaminants on Thayer-Martin plates, with the vancomycin-colistin-nystatin inhibitor implicated as the source of growth. In like manner, Otto and co-workers (10) described five clinical isolates of *Pseudomonas vesicularis* that had been recovered on Thayer-Martin medium after inoculation with cervical specimens. These were not ascribed to

contamination. Although 4 of the 12 cultures described in this report were recovered on modified Thayer-Martin agar, the diversity of specimens representing the sources of isolates ruled out modified Thayer-Martin or vancomycin-colistin-nystatin inhibitor as a provable source of contamination.

The uniform resistance of *Pseudomonas* sp. 1 and 2 to colistin accounts for their recovery on modified Thayer-Martin medium. *Pseudomo-*

TABLE 4. Susceptibility of colistin-resistant pseudomonads by disk diffusion and minimal inhibitory concentration tests^a

Antibiotic	<i>Pseudomonas</i> sp. 1 (5)		<i>Pseudomonas</i> sp. 2 (6)		CDC Va-1 (3)	
	K-B ^b	MIC ^c	K-B	MIC	K-B	MIC
Ampicillin	6	16->16	6	4-16	6	8->16
Carbenicillin	6	>512	6	32-512	10-27	64-512
Cephalothin	6-11	32-64	12-18	16-32	16-35	16-32
Chloramphenicol	6-12	32	13-17	32	17-24	8-32
Tetracycline	19-24	0.5-1.0	23-31	0.5-2.0	26-28	0.25-1
Kanamycin	6	64	6-19	16->64	22-30	4-8
Gentamicin	6	>16	6	>16	17-28	2-4
Tobramycin	6	>16	6	16->16	21-28	2
Amikacin	6	64	6	16->16	23	4-16

^a Colistin resistant at 4 µg/ml. Numbers within parentheses indicate the number of strains tested.

^b K-B, Kirby-Bauer disk diffusion method. Range of values given as zones of inhibition in millimeters.

^c MIC, Minimum inhibitory concentration. Range of values given as micrograms per milliliter.

nas IVd seemed to be one of the few nonfermentative bacteria that were resistant to the polymyxin class of antibiotics (6), although isolates tested in one study were susceptible to polymyxin (15). Reports of antibiotic susceptibility of the CDC Va group are scarce, although *P. cepacia*, *P. vesicularis*, and *Pseudomonas paucimobilis* (CDC-IHK-1) of the glucose-oxidizing pseudomonads have been shown to be resistant to polymyxin or colistin (9, 10).

Although a large number of strains of other closely related groups were not tested, the descriptions extant suggest that the Va group is diverse and may consist of several distinct organisms (13). The 12 strains comprising the heretofore undescribed organisms formed two homogeneous groups which could be distinguished from members of Va-1. At present, it is unknown how widespread the distribution of these organisms may be, or what their exact relationship is to the Va group. It is clear, however, that isolates tentatively designated as *Pseudomonas* sp. 1 and *Pseudomonas* sp. 2 are biochemically distinct from other organisms recovered from clinical specimens.

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