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Identification of *Vibrio hollisae* sp. nov. from Patients with Diarrhea

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The name *Vibrio hollisae* (synonym = Special Bacteriology group EF-13) is proposed for a new group of 16 strains that occurred in stool cultures of patients with diarrhea. *V. hollisae* is a small gram-negative rod, which is motile with a single polar flagellum. No lateral or peritrichous flagella were observed, even when it was grown on a solid medium. Sodium chloride is required for growth, so *V. hollisae* is a halophilic vibrio. Strains were positive (36°C, 24 or 48 h) for oxidase (Kovacs), indole production, nitrate reduction to nitrite, and fermentation of D-glucose (acid, no gas), L-arabinose, D-galactose, and D-mannose. Strains were negative for the following tests often used in enteric bacteriology: lipase (corn oil); deoxyribonuclease; gelatinase; methyl red; Voges-Proskauer; utilization of citrate, acetate, and malonate; L-lysine decarboxylase (Møllers); L-ornithine decarboxylase (Møllers); L-arginine dihydrolase (Møllers); growth in KCN medium; and acid production from D-adonitol, D-arabitol, cellobiose, dulcitol, erythritol, glycerol (25% delayed positive at 7 days), *i*-(myo)-inositol, lactose, maltose, D-mannitol, melibiose, α-methyl-D-glucoside, mucate, raffinose, L-rhamnose, salicin, D-sorbitol, sucrose, trehalose, and D-xylose. None of the strains was motile (semisolid medium) at 36°C at 48 h, but by 7 days 88% were motile. The strains did not grow within 2 days when plated on thiosulfate-citrate-bile salts-sucrose (TCBS) agar or MacConkey agar, but they grew on sheep blood agar and marine agar. By DNA-DNA hybridization (75°C, hydroxyapatite with ^{32}P), *V. hollisae* was only 0 to 4% related to 21 named species in *Vibrio* and *Photobacterium*. The type strain is designated ATCC 33564, which has a mean guanine-plus-cytosine content in DNA of 50 mol%. With the disk diffusion method *V. hollisae* had relatively large zones of inhibition around penicillin, ampicillin, carbenicillin, cephalothin, colistin, polymyxin B, streptomycin, kanamycin, gentamicin, tetracycline, chloramphenicol, and sulfadiazine. Future studies should focus on the isolation of this new vibrio and its ecology and relationship to human diseases.

Currently, the genus *Vibrio* has 20 described species (2), but fewer than half of the species have been associated with human disease. As late as 1975 only *Vibrio cholerae*, *V. parahaemolyticus*, and *V. alginolyticus* were considered human pathogens (6). *V. vulnificus* was described in 1976 as a cause of wound infections and fulminating septicemia (3, 11, 14), and in 1980 *V. fluvialis* (synonyms = group EF-6 and group F) was incriminated as a probable cause of diarrhea (15, 18). Within the last year, two new *Vibrio* species have been described, and both are associated with human infections. *V. mimicus* (8) probably causes diarrhea, and *V. damsela* (18a) causes skin lesions in marine fish and has also been isolated from human wound infections, where it may be the causative agent. In addition, *V. metschnikovii* (17), previously not believed to be a human pathogen, has recently been implicated as a cause of peritonitis and bacteremia (16). These reports bring the number of *Vibrio* species associated with human disease to eight. The purpose of this paper is to describe a new species, *V. hollisae* (synonym = Special Bacteriology group EF-13), which is a probable cause of human diarrhea.

MATERIALS AND METHODS

General. Unless exceptions are given, the following statements hold throughout this paper: the temperature of incubation was 36 ± 1°C; water refers to glass-distilled water; media were sterilized in an autoclave at 121°C for 15 min; and the term antibiotic refers to true antibiotics and to synthetic antimicrobial agents.

Nomenclature. The classification of *Vibrio* and *Photobacterium* recently proposed by Baumann et al. (2) is used, with the addition of the recently described

TABLE 1. List of *V. hollisae* strains studied

Enteric Section no.	Other no.	Clinical source	Location of sender
0921-79	ATCC 33565	Stool	Louisiana
0075-80[a]	ATCC 33564	Stool	Maryland
0519-81		Stool	Florida
0623-81		Stool	Louisiana
0624-81		Stool	Louisiana
0884-81		Stool	Florida
0886-81		Stool	Florida
9032-81	Special C6251[b]	Stool	Georgia
9033-81	Special D7980	Small intestine	North Carolina
9034-81	Special E1144	Stool	Maryland
9035-81	Special E5327	Stool	Louisiana
9036-81	Special E5615	Stool	Texas
9038-81	Special E8283	Stool	Pennsylvania
9039-81	Special F357	Stool	Florida
9041-81	Special D6984	Blood	Maryland
9042-81	Special D2733	Stool	Georgia

[a] Type strain for the species.
[b] Number assigned by the Special Bacteriology Section, CDC.

species *V. fluvialis* (18), *V. mimicus* (8), and *V. damsela* (18a). *V. hollisae* is proposed as a new species in this paper.

Bacterial strains. Sixteen strains of *V. hollisae* that had been received for identification at the Enteric Section and the Special Bacteriology Section, Centers for Disease Control (CDC), were studied. These strains and their sources are listed in Table 1. The type strain was designated ATCC 33564 (CDC no. 0075-80). All strains were grown on marine agar and frozen in 10% skim milk at −70°C and were also maintained at room temperature (18 to 28°C) in sealed tubes of "marine semisolid medium" (37.4 g of marine broth [Difco Laboratories, Detroit, Mich.], 4 g of agar, and 1,000 ml of water). Stock preparations of unlabeled DNA from 18 species of *Vibrio* and 3 species of *Photobacterium* previously prepared for an ongoing study were also used. These species included the type strains of *V. cholerae*, *V. parahaemolyticus*, *V. alginolyticus*, *V. anguillarum*, *V. vulnificus*, *V. damsela*, *V. harveyi*, *V. fischeri*, *V. fluvialis*, *V. metschnikovii*, *V. gazogenes*, *V. campbellii*, *V. natriegens*, *V. proteolyticus*, *V. splendidus*, *V. pelagius*, *V. nereis*, *V. nigripulchritudo*, *Photobacterium angustum*, *P. phosphoreum*, and *P. leiognathi* (2).

Media. Whenever possible, dehydrated media from commercial sources were used. Marine broth and marine agar were obtained from Difco Laboratories. The media used were generally those of Edwards and Ewing and have previously been described in detail (10, 12, 13). Most of the media used for standard tests contain 0.5% NaCl. Those media that do not contain any NaCl were supplemented with 1% NaCl. These media are methyl red, Voges-Proskauer (VP), L-lysine decarboxylase (Møller's), L-ornithine decarboxylase (Møller's), L-arginine dihydrolase (Møller's), gelatin, esculin broth, nitrate, and nutrient agar. Two media used to test for indole production, peptone water and heart infusion broth, which already contain 0.5% NaCl, were supplemented with an additional 0.5% NaCl so that the final NaCl concentration was 1%. Kovacs reagent (10) was used to test for indole. When "(1% NaCl)" follows the name of a medium, it means that NaCl was added to a final concentration of 1%. The modified O'Meara method as described by Hollis et al. (13) was used for the VP test, except that 1% NaCl had been added to the medium as previously stated. With this method the medium was inoculated, incubated for 2 days, and tested with 5% α-naphthol in ethyl alcohol (absolute) and 0.3% creatine in 40% KOH.

The medium used for the NaCl tolerance tests was nutrient broth (Difco) to which the following concentrations of NaCl were added: 0, 1, 3.5, 6, 8, 10, and 12% (wt/vol). The media were sterilized in volumetric flasks, and then a sufficient quantity of sterile distilled water was added to obtain the correct volume. These media were dispensed aseptically into sterile screw-cap tubes (8 ml; 16 by 125 mm) and stored at 5°C with the caps tightened.

Morphology of cells and colonies. Cellular morphology was observed on five strains, grown for 24 h in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.). Gram stains were done on air-dried cultures by Hucker's modified procedure (13). Flagella stains were done on four strains by the silver-plating method of West et al. (13). These strains were grown in flagella broth at 25°C. In addition, strain ATCC 33564 was grown on heart infusion agar at 25°C before flagella staining to determine whether it could also form lateral or peritrichous flagella (2).

Colonial morphology was determined by streaking all strains onto blood agar (Trypticase soy agar plus 5% defribrinated sheep blood), marine agar, and thiosulfate-citrate-bile salts-sucrose (TCBS) agar. Six strains were plated on MacConkey agar. The number of colonies and their size and shape were recorded, but plating efficiencies were not determined on these media.

Biochemical and miscellaneous tests. The standard set of biochemical tests (10, 12, 13) used by the Enteric Section was done on all strains (see Table 2).

The NaCl tolerance tests were done as follows: growth on a 24-h marine agar plate was removed with a

cotton swab and suspended in 2 ml of nutrient broth to the turbidity of a 0.5 McFarland standard; 1 drop (approximately 0.03 ml) of this suspension was added to each tube with a Pasteur pipette. The tubes were observed for 7 days, and the development of any visible turbidity was considered a positive test.

The string test, the test for sensitivity to 2,4-diamino-6,7,-diisopropylpteridine phosphate (vibriostatic compound O/129; Sigma Chemical Co., St. Louis, Mo.), and the test for bioluminescence were also done. The string test was done by suspending growth from a Typticase soy agar plate (25°C, 24 h) in 1 drop of 0.5% sodium deoxycholate in water. After 1 min the loop was touched to the drop and slowly raised. If a "mucoid string" formed between the drop and loop as it was raised, the strain was considered to have a positive string test (8). Susceptibility to O/129 was determined by streaking the Trypticase soy broth culture used for antibiograms onto Trypticase soy agar before applying the O/129 disk (prepared by drying filter paper disks saturated with 0.1% O/129 in acetone) and incubating for 24 h at 25°C. The zones of inhibition were measured in millimeters. All strains were grown on marine agar at 25°C for 24 h and examined in the dark for bioluminescence. Five minutes were allowed for eyes to become adapted to the dark before examining the cultures.

Antibiotic susceptibility tests. Antibiograms were done on all *V. hollisae* strains by the disk method of Bauer et al. (1) (see Table 3). The strains were grown in Trypticase soy broth for 5 to 6 h, diluted to the density of the 0.5 McFarland standard, and streaked onto Mueller-Hinton agar (with no added salt) before the disks were applied. The antibiotics are listed in Table 3. No minimal inhibitory concentrations were determined.

G+C content of DNA. The percentage of guanine plus cytosine (G+C) in DNA of the type strain, ATCC 33564, was calculated from the values for the midpoint of thermal denaturation, which was determined by the method described by De Ley (9), and the G+C ratio was calculated according to Colwell and Mandel (7).

DNA-DNA hybridization. DNA relatedness was determined on type strain ATCC 33564 and strain 0921-79. Unlabeled DNA was isolated and purified by methods previously described (4, 5). DNA from strain ATCC 33564 was labeled with ^{32}P in vitro by nick translation essentially by the methods of Rigby et al. (19) and the instructions furnished with a commercial nick translation reagent kit (catalog no. 8160; Bethesda Research Laboratories, Inc., Rockville, Md.). The relatedness of labeled DNA from strain ATCC 33564 to unlabeled DNAs of strain 0921-79 and the 21 *Vibrio* and *Photobacterium* species was determined on hydroxyapatite by methods previously described by Brenner and co-workers (5).

RESULTS

Morphology. Gram stains on five strains of *V. hollisae* indicate that it is a gram-negative rod with some cells slightly curved. They are approximately 0.5 μm wide by 1.5 to 2.0 μm long (dried and Gram stained). Cells have a single polar flagellum, but occasionally a subpolar flagellum has been observed (13). When the strain was grown on heart infusion agar, no lateral flagella were seen, in contrast to some *Vibrio* species (18). *V. hollisae* grew well on blood and marine agars. On blood agar, colonies were 1 to 2 mm in diameter at 24 h, with a weak zone of hemolysis around individual colonies. On marine agar the colonies were also 1 to 2 mm in diameter. Two colony types were often observed on blood and marine agars. One type was more opaque, but gave the same biochemical reactions. None of the strains grew on TCBS agar. Four of six strains did not grow when plated on MacConkey agar, but two strains grew very lightly in 3 days, in the heaviest area of streaking. The strains grew well at 25 and 36°C.

Biochemical reactions. The biochemical reactions obtained with the 16 *V. hollisae* strains are listed in Table 2 along with the reactions for the type strain. No growth was obtained with routine media (no added NaCl) for methyl red-VP, Møller decarboxylase, esculin, and nitrate; therefore, these are not listed in Table 2. Phenylalanine agar contains 0.5% NaCl, but no growth occurred on it and it is omitted from Table 2. The *V. hollisae* strains were positive for indole, oxidase, and nitrate with NaCl added and negative for VP, L-lysine and L-ornithine decarboxylases, and L-arginine dihydrolase; they produced acid from D-glucose, L-arabinose, D-galactose, and D-mannose. Strains did not grow in nutrient broth without NaCl, but did grow with 1, 3.5, and usually 6% NaCl (81% at 2 days). No bioluminescence was observed for any of the strains grown on marine agar at 25°C for 24 h. The sensitivity to O/129 was partial. An incomplete or hazy zone of inhibition was seen around the O/129 disk; however, no complete inhibition was observed. All strains gave a positive string test.

Antibiotic susceptibility tests. The results obtained with the disk susceptibility method are listed in Table 3. All strains had relatively large zones around all antibiotics tested, including penicillin and polymyxin B.

DNA hybridization. Strain ATCC 33564 was 98% related to 0921-79 at 60°C and 94% at 75°C with a divergence of 0.3%. It was only 0 to 4% related to the 21 *Vibrio* and *Photobacterium* strains tested at 75°C.

G+C ratio. The G+C content of DNA for the type strain was 49.3 to 51.0 mol% (mean = 49.8 mol%). The thermal denaturation midpoint was 89.5 to 90.2°C (mean = 89.7°C).

DISCUSSION

Biochemical tests and DNA relatedness studies indicate that strains previously called Special Bacteriology group EF-13 represent a new species in the genus *Vibrio*. *Vibrio hollisae* sp. nov.

is proposed as the name for this new species. The name was coined by F.W.H., J.J.F., and D.J.B. to honor Dannie G. Hollis for her contribution to knowledge of the fermentative and nonfermentative bacteria of medical importance. She was also the first to recognize group EF-13 as a separate group. The name was originally coined without the knowledge of D.G.H., who is an author of this paper. The species name, *hollisae*, is treated as a neo (modern)-Latin genitive noun meaning "of Hollis."

A description of *V. hollisae* sp. nov. follows. The cells are gram-negative rods, straight to slightly curved, and are about 0.5 μm wide by 1.5 to 2 μm long with a single polar flagellum. No lateral flagella were seen on flagellar stains of the type strain when it was grown on solid media. The strains are NaCl-requiring, oxidase-

TABLE 2. Biochemical reactions of 16 *V. hollisae* strains and the type strain

Test	Cumulative % positive[a] at:			Reaction for type strain ATCC 33564[b]
	1 day	2 days	7 days	
Indole		38		+
Indole (1% NaCl)		100		+
Methyl red (1% NaCl)		0		−
VP (1% NaCl)		0		−
Citrate (Simmons)	0	0	0	−
H_2S on triple sugar iron	0	0	0	−
Urea	0	0	0	−
L-Lysine (Møller's) (1% NaCl)	0	0	0	−
L-Arginine (Møller's) (1% NaCl)	0	0	0	−
L-Ornithine (Møller's) (1% NaCl)	0	0	0	−
Motility	0	0	88	+[7]
Gelatin (22°C)	0	0	0	−
Gelatin (1% NaCl)	0	0	0	−
KCN, growth in	0	0	0	−
Malonate	0	0	0	−
D-Glucose				
Acid	100	100	100	+
Gas	0	0	0	−
Acid from:				
Adonitol	0	0	0	−
L-Arabinose	94	94	100	+
D-Arabitol	0	0	0	−
Cellobiose	0	0	0	−
Dulcitol	0	0	0	−
Erythritol	0	0	0	−
D-Galactose	94	100	100	+
Glycerol	0	0	25	−
i-Inositol	0	0	0	−
Lactose	0	0	0	−
Maltose	0	0	0	−
D-Mannitol	0	0	0	−
D-Mannose	100	100	100	+
Melibiose	0	0	0	−
α-CH_3-glucoside	0	0	0	−
Raffinose	0	0	0	−
L-Rhamnose	0	0	0	−
Salicin	0	0	0	−
D-Sorbitol	0	0	0	−
Sucrose	0	0	0	−
Trehalose	0	0	0	−
D-Xylose	0	0	0	−
Esculin (1% NaCl)	0	0	0	−
Mucate	0	0	0	−
Tartrate (Jordan's)	44	81	81	−
Acetate	0	0	0	−
Lipase (corn oil)	0	0	0	−
DNase				
25°C	0	0	0	−
36°C	0	0	19	−

TABLE 2—*Continued*

Test	Cumulative % positive[a] at:			Reaction for type strain ATCC 33564[b]
	1 day	2 days	7 days	
$NO_3^- \rightarrow NO_2^-$ (1% NaCl)	100			+
Oxidase (Kovacs)	100			+
Oxidase (1% NaCl)	100			+
ONPG[c]	0	0	0	−
Citrate (Christensen's)	0	0	0	−
Tyrosine clearing	0	0	0	−
String test	100			+
Growth in nutrient broth plus:				
0% NaCl	0	0	0	−
1% NaCl	100	100	100	+
3.5% NaCl	100	100	100	+
6% NaCl	69	81	88	$+^5$
8% NaCl	0	0	0	−
10% NaCl	0	0	0	−
12% NaCl	0	0	0	−

[a] A blank space indicates not determined.
[b] Symbols: +, positive at 24 h or at time of the test; −, negative at end of appropriate incubation period or 7 days; superscript gives day the reaction became positive.
[c] ONPG, *o*-Nitrophenyl-β-D-galactopyranoside.

positive, nitrate-positive fermenters. All are indole positive and produce acid from D-glucose, L-arabinose, D-galactose, and D-mannose. They are slowly and weakly motile (88% at 7 days) at 36°C; the type strain was also slow and weak when tested at 25°C. The following tests were negative: methyl red, VP, Simmons citrate, urea, L-lysine and L-ornithine decarboxylase, L-arginine dihydrolase, gelatin hydrolysis, and fermentation of most sugars and polyhydroxyl alcohols. The G+C content for the type strain is 49.8 mol%. This organism has most commonly been isolated from stool specimens of persons with diarrhea. The strains had relatively large zones around all antibiotics tested (agar diffusion). A more detailed description of the species and its type strain is given in Table 2. The type strain (holotype) is designated ATCC 33564 (CDC 0075-80) and was isolated from the feces of a patient in Maryland.

TABLE 3. Susceptibility of 16 *V. hollisae* strains by agar diffusion

Antibiotic[a]	Zone diam (mm)		
	Range	Mean	SD
Ampicillin (10)	23–31	28	2.2
Carbenicillin (100)	23–36	32	3.3
Cephalothin (30)	20–29	24	2.2
Chloramphenicol (30)	30–35	32	1.7
Colistin (10)	13–16	14	1.0
Gentamicin (10)	20–26	24	1.6
Kanamycin (30)	18–26	23	1.9
Nalidixic acid (30)	25–31	29	1.8
Penicillin (10 U)	20–30	25	2.9
Polymyxin B (50 U)	11–15	12	1.2
Streptomycin (10)	16–20	18	1.2
Sulfadiazine (250)	12–26	17	3.5
Tetracycline (30)	18–27	25	2.2

[a] Number in parentheses is the disk concentration in units for penicillin and polymyxin B and in micrograms for all other antibiotics.

The biochemical reactions that differentiate *V. hollisae* from other clinically significant NaCl-requiring *Vibrio* species are given in Table 4. *V. hollisae* can be differentiated because it is strongly indole positive, is negative for L-lysine, L-arginine, and L-ornithine, and ferments L-arabinose. Percentages given in Table 4 for indole production refer to results obtained with heart infusion broth (1% NaCl) and Kovacs reagent. *V. cholerae* and *V. mimicus* were not included in Table 4 because they do not require added NaCl.

V. hollisae was only slightly related by DNA hybridization to 17 of the 20 currently recognized species of *Vibrio* and the three species of *Photobacterium*. It was not tested against *V. mimicus, V. marinus, V. costicola*, or *V. logei* (2); however, the phenotypic descriptions given for these species are quite different from that of *V. hollisae*. These species will be tested in the future.

An unusual finding was that *V. hollisae* did not grow (1 or 2 days, 36°C) on TCBS or MacConkey agars, which are frequently used as plating media for stool cultures from people with diarrhea. However, the Special Bacteriology Section at CDC found that when MacConkey

TABLE 4. Differentiation of *V. hollisae* from other halophilic *Vibrio* species isolated from human clinical specimens[a]

Test	% Positive in 48 h						
	V. hollisae (*n* = 16)	*V. damsela* (*n* = 16)	*V. parahaemolyticus* (*n* = 65)	*V. alginolyticus* (*n* = 28)	*V. vulnificus* (*n* = 51)	*V. fluvialis* (*n* = 20)	*V. metschnikovii* (*n* = 17)
Indole (1% NaCl)	100	0	88	50	92	17	24
VP (1% NaCl)	0	100	0	93	0	0	100
L-Lysine (Møller's) (1% NaCl)	0	56	100	100	98	0	24
L-Arginine (Møller's) (1% NaCl)	0	100	0	0	0	92	53
L-Ornithine (Møller's) (1% NaCl)	0	0	82	65	57	0	0
Acid from:							
L-Arabinose	94	0	80	3	0	89	0
Cellobiose	0	0	2	3	100	39	12
Lactose	0	0	0	0	95	4	59
Maltose	0	100	99	100	100	100	100
D-Mannitol	0	0	100	100	41	96	100
Salicin	0	0	0	0	100	0	12
Sucrose	0	0	3	97	14	100	100
$NO_3 \rightarrow NO_2$ (1% NaCl)	100	100	100	100	100	100	0
Oxidase (1% NaCl)	100	94	100	100	100	100	0

[a] Data taken from data base of Enteric Section, CDC.

agar slants were inoculated with heart infusion broth cultures of *V. hollisae*, 58% grew in 48 h (75% in 7 days). *V. hollisae* grew well on sheep blood agar and marine agar. Primary isolation from clinical specimens or the environment has not been attempted, and we have no information as to how it was achieved. If the organism does not grow on MacConkey agar, it would probably not grow on more selective media such as Hektoen and salmonella-shigella agars. In a study of the clinical characteristics and epidemiology of disease associated with *V. hollisae*, it was found (H. G. Miller, CDC, personal communication) that five of nine strains were isolated in pure culture from stool specimens of patients with diarrhea. Its clinical significance in eight cases has been studied and will be reported elsewhere. *V. hollisae* has been implicated as a possible cause of diarrhea in people who have eaten raw seafood. If TCBS agar cannot be used to isolate this organism from stools, it might be detected by flooding a blood agar plate with oxidase reagent and searching for oxidase-positive colonies. More data are needed on the isolation of this new organism from clinical specimens and the environment. We hope others will try to isolate this organism so that more can be learned about its ecology, epidemiology, and role in human disease.

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