JOURNAL OF CLINICAL MICROBIOLOGY, June 1991, p. 1206–1210
0095-1137/91/061206-05$02.00/0
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Vol. 29, No. 6

Aeromonas trota sp. nov., an Ampicillin-Susceptible Species Isolated from Clinical Specimens

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Received 10 January 1991/Accepted 21 March 1991

Previous DNA hybridization studies established 12 *Aeromonas* genospecies, from which nine phenotypic species have been proposed: *Aeromonas hydrophila*, *A. sobria*, *A. caviae*, *A. media*, *A. veronii*, *A. schubertii*, *A. salmonicida*, *A. eucrenophila*, and *A. jandaei*. We have delineated a new *Aeromonas* genospecies, *A. trota*, on the basis of 13 strains isolated primarily from fecal specimens from southern and southeastern Asia. All strains were highly related to the proposed type strain, AH2 (ATCC 49657^T): 51 to 100% (60°C) and 49 to 99% (75°C), with 0.2 to 2.2 divergence. AH2 was only 16 to 41% (60°C) related to all other *Aeromonas* type strains and DNA group definition strains. The unique profile of *A. trota* includes negative reactions for esculin hydrolysis, arabinose fermentation, and the Voges-Proskauer test, positive reactions for cellobiose fermentation, lysine decarboxylation, and citrate utilization, and susceptibility to ampicillin, as determined by the broth microdilution MIC method and the Bauer-Kirby disk diffusion method (10 μg). Nine of the *A. trota* strains were from a single study of 165 geographically diverse aeromonads. This finding questions the efficacy of screening fecal specimens for *Aeromonas* spp. with ampicillin-containing media and suggests a previously unrecognized prevalence of this new species.

The genus *Aeromonas* consists of nine validated and/or proposed species: *A. hydrophila*, *A. sobria*, *A. caviae*, *A. salmonicida*, *A. media*, *A. veronii* (biogroups sobria and veronii), *A. schubertii*, *A. eucrenophila*, and *A. jandaei* (1, 2, 7, 13, 14, 16, 18, 23, 24). They are all oxidase-positive, glucose-fermenting, facultatively anaerobic gram-negative rods that are resistant to vibriostatic agent 0/129, reduce nitrates to nitrites, are unable to grow in 6.5% NaCl, and are generally resistant to ampicillin and carbenicillin. All species are motile via a polar flagellum, except for most *A. salmonicida* strains (26).

These organisms are globally distributed and autochthonous to marine, estuarine, and freshwater environments (12, 19). They can tolerate temperatures ranging from 4 to 42°C, accounting for their ability to cause disease in a wide variety of cold- and warm-blooded animals (16). Although they are currently classified in the family *Vibrionaceae*, it was proposed in 1986 by Colwell et al. that, on the basis of 5S rRNA cataloging, 5S RNA sequencing, and RNA-DNA hybridization data, the aeromonads constituted a separate family, *Aeromonadaceae* (10).

All of the more recently described species, with the exception of *A. eucrenophila*, have been associated with human disease (2). The purpose of this study was to establish the taxonomic position and possible clinical significance of an unusual group of ampicillin-susceptible *A. sobria*-like organisms that thus far appear to be predominantly from southern and southeastern Asia.

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MATERIALS AND METHODS

Bacterial strains. The thirteen strains of *A. trota* used in this study are listed in Table 1 along with their geographic distribution, clinical source of isolation, and original phenotypic designation when received. All strains were originally isolated on media that did not contain antibiotics, in particular, ampicillin. The strains were then frozen in 2 ml of tryptic soy broth with 10% glycerol (Remel, Lenexa, Kans.), maintained at −70°C, and subsequently subcultured to tryptic soy agar plates (BBL Microbiology Systems, Cockeysville, Md.). After overnight incubation at 36 ± 1°C, single colonies were subcultured as stated above and used for all analyses. Unless otherwise stated, all analyses were performed at 36 ± 1°C.

Phenotypic analysis. Of the 32 identification tests (see Table 2), 30 were performed either by conventional methods (15, 22) with media and biochemicals from Remel or by the API 20E rapid identification system (Analytab Products, Plainview, N.Y.). Other media used for identification and characterization were modified GCF agar for the detection of H_2S from cysteine (25) and BEAM elastase agar (11).

DNA-related analysis. DNA was extracted and purified by previously described methods (4, 5) from the 13 *A. trota* strains, 1 *Vibrio parahaemolyticus* strain, and all type and reference strains representing all recognized *Aeromonas* DNA hybridization groups. The proposed type strain, ATCC 49657^T (AH2), was nick translated and labeled with ^{32}P (NEK-004; Dupont, NEN Research Products, Boston, Mass.), and hybridization analysis was performed at both the optimal reassociation temperature of 60°C and the stringent reassociation temperature of 75°C. Relatedness was expressed as the relative binding ratio and as the divergence in the melting temperature (dTm) (see Table 3). The relative binding ratio was calculated as a percentage to enable the determination of similarities between strains. The relative

TABLE 1. *A. trota* strains studied

Strain	ATCC no.	Geographic location	Source	Original designation
AH2	49657	India	Stool	*A. hydrophila* or *A. sobria*
MOB/A7		Bangladesh	Stool	*A. sobria*
MOB/A8	49658	Bangladesh	Stool	*A. sobria*
MOB/A9		Bangladesh	Stool	*A. sobria*
MOB/A11		Bangladesh	Stool	*A. hydrophila* or *A. sobria*
MOB/A16		Bangladesh	Stool	*A. hydrophila*
AMC 5439		Maryland	Stool	*A. hydrophila*
NMRI-206		Indonesia	Stool	*A. sobria*
NMRI-208	49661	Indonesia	Stool	*A. sobria*
CHAK 5258		Indonesia	Stool	*A. caviae*
CHAK 5274		Indonesia	Stool	*A. caviae*
AS 66	49660	Thailand	Stool	*A. sobria*
AS 370	49659	California	Appendix	*A. sobria*

binding ratio values of approximately 70% (for reactions at 60°C) and 55% (for reactions at 75°C) and a dTm of ≤5°C were considered the cutoff values for DNA relatedness at the species level (27).

Antimicrobial susceptibility. The 13 *A. trota* strains were tested for their susceptibility to 17 antimicrobial agents by the broth microdilution MIC method with the Gram Negative Flex 1 and Flex 2 panels of the Vitek system (Vitek Systems, Hazelwood, Mo.). MICs for 50% of all strains (MIC_{50}) were calculated (see Table 5). Antibiotic susceptibility was also determined on Mueller-Hinton agar by the disk method of Bauer et al. (3), and percent susceptibility was calculated (see Table 5).

RESULTS AND DISCUSSION

Phenotypic analysis. Nine of the 13 *A. trota* strains were originally part of a large systematic assessment of 165 geographically diverse aeromonads from clinical, environmental, and veterinary sources (6). Cluster analysis by SAS/TAXAN, a numerical taxonomy program, revealed clusters representing the four major mesophilic species, *A. hydrophila*, *A. caviae*, *A. veronii* (biogroups sobria and veronii), and *A. schubertii*, and a small cluster of nine strains that have recently been proposed to be *A. jandaei* (formerly DNA group 9 *A. sobria*) (7). This numerical taxonomy analysis also revealed a second small cluster of nine strains that were distinct from all other *Aeromonas* phenotypic species. They were uniformly negative for esculin hydrolysis, arabinose fermentation, and acetoin utilization (Voges-Proskauer) and uniformly positive for citrate utilization and lysine decarboxylation. They were also generally positive for the production of gas from glucose and H_2S from cysteine on modified GCF media and generally negative for sucrose fermentation (Table 2). The discovery of ampicillin susceptibility in conjunction with the acquisition of four additional such strains (AS 66, AS 370, 5258, and 5274) led to further investigation of this cluster. Additional phenotypic markers, including failure to oxidize gluconate, positive cellobiose fermentation, and susceptibility to carbenicillin, led us to suspect that this cluster represented a new *Aeromonas* phenotypic species.

DNA hybridization. Definitive identification of this cluster as a new species was accomplished in a stepwise process. Strain AH2 was labeled and tested against all the *Aeromonas* type and definition strains and ATCC reference strains

TABLE 2. Biochemical reactions of 13 *A. trota* strains

Test	Cumulative % positive on day:				Reaction for type strain ATCC 49657[T] (AH2)[a]
	1	2	3	7	
Indole production	100				+
Voges-Proskauer		0			−
Citrate[b]	100				+
Esculin hydrolysis			0		−
H_2S on TSI[c]				0	−
H_2S on modified GCF media	75		100		+
Lysine (Moeller's)	100				+
Arginine (Moeller's)	100				+
Ornithine (Moeller's)			0		−
Motility (25°C)	92				+
Gelatin hydrolysis	100				+
D-Glucose:					
Acid	100				+
Gas	75	83	83		+
Acid produced from:					
Amygdalin[b]	83				+
L-Arabinose			0		−
Cellobiose	100				+
Inositol[b]	0				−
D-Mannitol	75	83	83		+
Mannose	100				+
L-Rhamnose[b]	0				−
Salicin			0		−
D-Sorbitol[b]	0				−
Sucrose	17	17	17	17	−
Gluconate		0			−
Ascorbate utilization		0			−
Elastase				0	−
Nitrate reduction	100				+
Oxidase	100				+
ONPG[d]	100				+
String test	0				−
0/129 Resistance	100				+
Hemolysis on SBA[e]	54	77	77		+

[a] −, negative at end of appropriate incubation period; +, positive at 24 h or at end of appropriate incubation period.
[b] API 20E result.
[c] TSI, triple sugar iron.
[d] ONPG, *o*-nitrophenyl-β-galactopyranoside.
[e] SBA, sheep blood agar (tryptic soy agar with 5% sheep erythrocytes).

(Table 3) and found to exhibit only 16 to 41% homology. The second run used AH2 as the labeled probe against the other 12 phenotypically similar strains (Table 1). They showed 51 to 100% homology at 60°C and 49 to 99% homology at the more stringent 75°C, with a dTm of 0.2 to 2.2 (Table 3). Although strains A16, AMC 5439, and A7 fell below the 60°C accepted cutoff of 70% homology, their respective values for dTm and percent homology at 75°C taken collectively confirm that they are of the same species (27).

Designation of *A. trota*. We propose the name *Aeromonas trota* for the 13 strains listed in Table 1 (21). The species name (pronounced trō′ta) is derived both from the Greek adjective "trotos," meaning "vulnerable," and the modern (neo) Latin feminine adjective "trota," meaning "vulnerable." This designation is predicated upon the susceptibility of the organisms to ampicillin and carbenicillin, which first led us to suspect that the organisms were a separate group of aeromonads. The type strain of the species is designated ATCC 49657[T] (formerly AH2), and a complete description of all of the strains is provided in Table 2. The unique profile of *A. trota* includes negative reactions for esculin hydrolysis,

TABLE 3. DNA relatedness of the type strain of *A. trota* to other strains of *A. trota* and *Aeromonas* spp.

Strain	Relatedness (%) to labeled DNA of *A. trota* ATCC 49657^T		
	60°C	dTm[a]	75°C
ATCC 49657^T (AH2)	100	0.0	100
MOB/A7	67	0.2	71
MOB/A8	81	1.6	79
MOB/A9	100	2.0	93
MOB/A11	73	0.9	73
MOB/A16	69	2.2	68
AMC 5439	51	1.0	49
NMRI-206	100	1.0	98
NMRI-208	82	0.4	88
CHAK 5258	100	2.2	92
CHAK 5274	85	1.7	81
AS 66	82	0.3	89
AS 370	91	1.0	99
V. parahaemolyticus	14	11.1	ND[b]
A. hydrophila (DNA group 1) CDC 9079-89 (ATCC 7966^T)	35	ND	ND
A. sobria (DNA group 7) CDC 9538-76 (CIP 7433^T)	32	ND	ND
A. veronii biogroup sobria (DNA group 8)			
CDC 9080-79 (ATCC 9071)	36	ND	ND
CDC 0437-84	36	ND	ND
A. veronii biogroup veronii (DNA group 10) CDC 1169-83 (ATCC 35624^T)	41	ND	ND
A. hydrophila (DNA group 2) CDC 9533-76	37	ND	ND
A. hydrophila (DNA group 3) CDC 0434-84	27	ND	ND
A. caviae (DNA group 4) CDC 9083-79 (ATCC 15468^T)	30	ND	ND
A. caviae (DNA group 5a) CDC 0862-80	38	ND	ND
A. media (DNA group 5b) CDC 9072-83	33	ND	ND
A. eucrenophila (DNA group 6) CDC 0859-83 (NCMB 74^T)	32	ND	ND
A. jandaei (DNA group 9) CDC 0787-80 (ATCC 49568^T)	35	ND	ND
A. schubertii (DNA group 12) CDC 2446-81 (ATCC 43700^T)	17	ND	ND
Aeromonas group 501 CDC 2478-85	16	ND	ND
Enteric group 77 (DNA group 11) CDC 1306-83	32	ND	ND

[a] dTm, difference in melting temperature between each homologous and heterologous DNA complex.
[b] ND, not determined.

arabinose fermentation, Voges-Proskauer test, and gluconate oxidation. Further, *A. trota* is positive for cellobiose fermentation, lysine decarboxylation, and citrate utilization but is susceptible to both ampicillin and carbenicillin. A comparison of this profile with those of other mesophilic *Aeromonas* species is given in Table 4 (6, 7, 13–15, 17, 23, 26). Negative sucrose fermentation, positive mannitol fermentation, gas production from glucose, production of H_2S from modified GCF media, and hemolysis on tryptic soy agar with 5% sheep blood could also be used as presumptive markers but showed slight variability within the group and were not as definitive as the profile markers described above.

API 20E profile numbers for these strains identified them all as "*A. hydrophila* complex," with an identification level of "good" to "excellent," but with the comment that the negative reaction for mannitol and/or sucrose was considered atypical in their data base. Likewise, these strains were all identified as *A. hydrophila* in the Vitek system with a Gram Negative Identification (GNI) Card at 99% accuracy.

Antimicrobial susceptibility. The antibiogram of *A. trota* generally showed susceptibility to all 17 antimicrobial agents tested, with the exception of moderate susceptibility to cefoxitin and cefazolin and generalized resistance to cephalothin. This resistance to cephalothin is atypical of clinical *A. sobria* (now *A. veronii* biogroup sobria) (17), although the newly proposed *A. jandaei* (formerly DNA group 9 *A. sobria*) also showed generalized resistance to cephalothin (7). This difference may serve as a useful marker for the differentiation of one *A. sobria* biogroup from another.

Most interesting was the uniform susceptibility of *A. trota* to ampicillin and carbenicillin found by both the broth microdilution MIC method and the classic Bauer-Kirby disk diffusion method (Table 5). The absence of plasmids in the type strain, ATCC 49657^T (8), and in aeromonads in general supports the observation that resistance to ampicillin and carbenicillin, when observed, is most likely chromosomal in nature (9, 20).

The majority of the strains were isolated during the mid-1970s and early 1980s, well before the availability and resultant trend toward the use of ampicillin-containing media for the screening and isolation of aeromonads. This fact suggests that the use of such media may result in a negative selection against *A. trota*, which has apparently been in the environment for quite some time and appears to be capable of causing human disease. Awareness of ampicillin susceptibility is important not only for *A. trota* but also for other aeromonads as well, since results from the previously mentioned numerical taxonomy study at the University of Maryland also revealed the existence of a subset of ampicillin-susceptible *A. caviae* strains from clinical specimens (6).

Geographic distribution. Another interesting aspect of this new species is its geographic distribution. Although it was found in five countries on two continents, a majority of the strains (11 of 13) originated from southern or southeastern Asia (Table 1). Whether this geographic distribution represents an original reservoir for *A. trota* in that area of the world that is now spreading to other areas can only be determined by both accurate biotyping and examination of antibiograms of a larger number of similar isolates from numerous geographic areas.

Clinical significance. *A. trota* was isolated almost exclusively from fecal specimens, with the exception of AS 370, which was isolated from an appendix. Although there is a strong association between aeromonads and gastrointestinal disease, Koch's postulates have not been fulfilled to date.

Most of the isolates in this study were accompanied by very limited patient information (Table 1), but there was a well described case history available on the type strain, ATCC 49657^T (AH2). The patient was a 28-year-old laboratory worker with no preexisting health problems. The individual was exposed to a brain heart infusion broth suspension of AH2 by accidental ingestion of an 18- to 24-h broth culture (approximately 10^9 organisms) while preparing it for permanent storage and subsequently suffered a severe bout of diarrhea with "rice-water" stools over a period of 2 days. The clinical symptoms arose suddenly, without prodromes, within 24 h of the laboratory accident. The illness was self-limiting and subsided uneventfully after 2 days without any further complications.

A stool culture was performed on two separate occasions during the illness by streaking fecal material on human blood agar, MacConkey agar, and chocolate agar plates and inoculating brain heart infusion broth. After overnight incubation at 30°C, pure cultures of a single type of gram-negative bacterium were observed on all the media. The organism

TABLE 4. Comparison of distinguishing profiles of *A. trota* and other *Aeromonas* spp.

Test	Result[a] for:						
	A. trota	*A. jandaei*	*A. veronii* biogroup sobria	*A. veronii* biogroup veronii	*A. schubertii*	*A. hydrophila*	*A. caviae*
Esculin hydrolysis	−	−	−	+	−	+	+
Voges-Proskauer	−	+	+	+	V	+	−
Cellobiose	+	−	−	+	−	−	V
Gluconate oxidation	−	+	+	ND	−	+	−
Fermentation of:							
Arabinose	−	−	−	−	−	V	+
Mannitol	+	+	+	+	−	+	+
Sucrose	−	−	+	+	−	+	+
Susceptibility to:							
Ampicillin	S	R	R	R	R	R	R
Carbenicillin	S	R	R	R	R	R	R
Cephalothin	R	R	S	S	S	R	R
Colistin[b]	S	R	S	S	S	V	S
Decarboxylase:							
Lysine	+	+	+	+	+	+	−
Arginine	+	+	+	−	+	+	+
Ornithine	−	−	−	+	−	−	−
Indole	+	+	+	+	−	+	+
H_2S on modified GCF media	+	+	+	+	−	+	−
Glucose (gas)	+	+	+	+	−	+	−
Hemolysis (5% sheep erythrocytes)	V	+	+	+	+	+	V

[a] −, negative; +, positive; V, variable; ND, not determined; S, susceptible; R, resistant.
[b] Single-dilution MIC, 4 μg/ml.

isolated on both occasions was oxidase positive, was beta-hemolytic on human blood agar, and was an opaque, nonlactose fermenter on MacConkey agar. Biochemical characterization with the API 20E system identified the organism as *A. hydrophila*, and further identification of the organism to the species level by the criteria of Popoff and Veron (23) assigned it to the *A. sobria* group. These isolates were preserved and subsequently confirmed to be identical to the original AH2 strain that was ingested. This was accomplished with the phenotypic analyses listed in Table 2.

Strain AH2 also exhibited mannose-sensitive hemagglutination and was untypeable by the Sakazaki serotyping scheme. Furthermore, sterile, filtered culture supernatants were found to possess hemolytic activity on human erythrocytes, cytolytic activity on Vero cells, and proteolytic activity on tryptic soy agar with added skim milk.

TABLE 5. Antimicrobial susceptibility of 13 *A. trota* strains

Antibiotic	Broth microdilution MIC method			Bauer-Kirby disk diffusion method	
	MIC		Interpretation	Amt/disk (μg)	% Susceptibility
	Range (μg/ml)	50% (μg/ml)[a]			
Amikacin	<2–8	4	S	30	100
Ampicillin	<0.25–2	<0.25	S	10	100
Aztreonam	<8	<8	S	30	100
Carbenicillin	<16	<16	S	100	100
Cefazolin	<8–>32	16	MS	30	62
Cefotetan	<16	<16	S	30	100
Cefoxitin	<2–16	16	MS	30	92
Ceftazidime	<8	<8	S	30	100
Ceftriaxone	<8	<8	S	30	100
Cefuroxime	<4	<4	S	30	100
Cephalothin	<2–>32	>32	R	30	8
Ciprofloxacin	<0.5	<0.5	S	5	100
Gentamicin	<0.5–25	15	S	10	100
Imipenem	<4	<4	S	10	100
Mezlocillin	<16	<16	S	75	100
Piperacillin	<8	<8	S	100	100
Tetracycline	<1–8	<1	S	30	92
Ticarcillin	<16	<16	S	85	100
Ticarcillin-clavulanate	<16	<16	S	75	100
Tobramycin	<0.05–2	1	S	10	100
Trimethoprim-sulfamethoxazole	<10	<10	S	23.75/1.25	100

[a] 50%, MIC for 50% of all strains tested.

Conclusions. We have reported on the isolation and identification of a new *Aeromonas* species, designated *A. trota*. This species has a biochemical profile of negative reactions for esculin hydrolysis, arabinose fermentation, Voges-Proskauer test, and gluconate oxidation, positive reactions for cellobiose fermentation, citrate utilization, and lysine decarboxylation, and a uniform dual susceptibility to ampicillin and carbenicillin. Rapid identification systems, such as API 20E, and automated identification systems, such as the Vitek system, will identify these isolates as either *A. hydrophila* complex or *A. hydrophila*. However, some of the distinguishing biochemical markers mentioned above are incorporated within these two systems and should lead the clinical microbiologist to suspect the possibility of *A. trota*.

Although *A. trota* appears to be predominantly from southern and southeastern Asia, it may in fact be present worldwide. Documentation of its possible clinical significance and virulence potential for humans, presented here, strongly suggests that this species should be considered in biotyping of aeromonads from clinical and environmental sources.

ACKNOWLEDGMENTS

We thank J. J. Farmer III for donation of several of the DNA group definition strains and Thomas MacAdoo for consultation on the derivation of the new species name. We acknowledge the supplies provided by Lisette Gonzalez (Analytab Products, Plainview, N.Y.) and Vitek Systems (Hazelwood, Mo.). We also thank Donna Watsky for technical assistance and Sharon Payne and Nancy Cox for typing the manuscript.

This work was done with the support of Agency for International Development grant DPE-5542-G-SS-7029-00.

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