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Aeromonas haianensis, Sp. Nov., from Eels and Humans

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Abstract: Ten strains of unidentified bacteria were isolated from diseased eels and patients with diarrhea in Haian County, Jiangsu Province. All isolates were Gram-negative motile rods growing in non-salt peptone medium at 37°C. They were oxidase positive, glucose fermenting and produced a brown pigment. The bacteria were resistant to O/129. The bacteria were identified as belonging to *Aeromonas*; some phenotype characteristics were different from these of the reported *Aeromonas* species. DNA-DNA hybridization studies showed that DNA of strain CCTCC AB97026 was 93 and 89% similar to that of strain 95-72, 96-109 and showed 63% relatedness to *Aeromonas veronii*, which is below the cut-off value for species differentiation. The determination of mol% G+C, DNA-DNA hybridization and 16S rRNA gene sequencing and fatty acids analysis indicated that these bacteria constitute a new *Aeromonas* species. These bacteria were susceptible to norfloxacin, furazolidone, nutgall and rhubarb and resisted to penicillin and SMZ. The LD_{50} for mice and eel is 3.9×10^{6} and 1.2×10^{5} cfu, respectively. The bacterium had some virulence factors, such as type IV pilli, HEC toxin and extracellular proteinase. All the above data and the epidemiological information indicated that the new bacterium was pathogenic to eel and humans. For the new species, the name of *Aeromonas haianensis* has been proposed because the bacteria were all isolated from Haian County, Jiangsu Province, China. The type strain is CCTCC AB97026^{T} (=IFO16641=strain95-173).

Key words: *Aeromonas haianensis* sp.nov., DNA-DNA hybridization, 16S rRNA gene, fatty acids analysis, Pathogenicity

INTRODUCTION

Aeromonads constitute a substantial part of the microbial flora in various types of freshwater and estuarine environments that have been implicated in the aetiology of a variety of systematic and localized diseases in various mammals, reptiles, fish and humans. The aeromonads were divided into two groups and four species (Popoff, 1984). The first group included psychrophylic and non-motile aeromonads, i.e. *A. salmonicida* and its three subspecies. The second group consisted of *A. hydrophila, A. caviae* and *A. sobria*, which were motile and mild temperature loving. Many new species have been described in recent years. Until now the number of species in the genus *Aeronomas* have increased to 16, including several subspecies (http://www.sv.cict.fr/bacterio), (Martinez-Murcia *et al*., 1992; Ali *et al*., 1996; Popoff, 1984; Esteve *et al*., 1995; Schubert *et al*., 1990; Schubert and Hegazi, 1988; Schubert *et al*., 1990; Carnahan *et al*., 1991; Allen *et al*., 1983; Huys *et al*. 1997b; Hickman-Brenner *et al*., 1988; Popoff and Véron, 1976; Carnahan *et al*., 1991; Hickman-Brenner *et al*., 1987; Pidiyar, 2002). Eel's death occurred in thr-ee ee-l, fishponds from 1995 in Haian County and the strains of *Aeromonas* were isolated without identifying them into definite species (Wang *et al*., 1998). These *Aeromonas* strains have been characterized by a polyphasic approach leading to the proposition of a new species *Aeromonas haianensis*.

The diseased eels (100-500 g), (*Anguilla japonica*), were collected from three eel farms in Haian County, Jiangsu Province. The eels were biopsied and the fluids from stomachs and intestines were collected; livers and kidneys samples were also taken for isolation of pathogenic bacteria. Stools of patients with diarrhea were also used for the isolation of the casual pathogen. Ten isolates were characterized 95-72, 95-73, 95-173, 95-174, 95-178, 96-109, 96-110 and 97-14 from the eels and 98-15, 03-37 from human stools. A total of 14 type strains were used for the comparison of *Aeromonas haianensis* with the other *Aeromonas* species.

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The ten new *Aeromonas* isolates were examined for 80 characteristics including morphological, physiological, biochemical nutritional and antimicrobial susceptibility tests. The methodology used was as described by Popoff (1984).

The mol% G+C content of the genomic DNA was determined from the midpoints (Tm) of the thermal denaturation profiles (Owen and Hill, 1979) DNA from *E.coli* K12 was used as control.

DNA-DNA hybridization was carried out by the microplate hybridization method described by Ezaki *et al.* (1990) and Jiao *et al.* (2001), i.e., DNAs from type strains were fixed on the microwells and the DNA from the strain to be identified was labeled with photobiotin, the hybridization was carried out at 30°C for 6 h and after washing step the hybridization rate was determined by colorization comparison.

Fatty Acids Methyl Esters (FAMEs) were extracted by following the method recommended by Sherlock Microbial Identification System protocol (MIS, MIDI Inc., Newark, Del). FAMEs were analyzed by gas chromatography with a Hewlett-Packard module HP 6890 instrument and the GC settings followed the MIS Operation Manual (MIDI, 1997). The FAMEs were identified and quantitated by the Sherlock MIS software (ver 2.95). The relative amount of each single fatty acid in a strain was expressed as a percentage of the total acids in the profile of that strain. The CFAs composition data were arranged as a raw matrix and dendrogram of experimental strains was generated by Statistica software package (ver 5.0, StatSoft. Inc., Tulsa, OK). Hierachical clustering analysis was performed using Unweighted Pair-Group Arithmetic Average (UPGMA) method by Euclidean distance coefficient.

Chromosomal DNA was extracted from cells and purified by using a rapid NaI-glass beads procedure described by Room *et al.* (1990). The 16Sr DNA genes of the novel isolates were amplified by PCR using conserved primers (8UA and 1458B) located on both ends of the target gene, 16S rRNA sequence was customary determined by Katoso Co. Using the dideoxynucleotide chain termination method. The sequence was aligned with *Aeromonas* 16S rRNA sequences retrieved from DDBJ (http://www.ddbj.nig.ac.jp) by using DNAstar software and the derived tree was reconstructed by DNAstar software.

The virulence of the isolated strain for eels and mice was examined. Eels (10-15 g) and 6-weeks old-mice (16-20 g) were used in the animal experiments. Each group with 6 animals were injected intraperitoneally with 0.2 mL of 18h cultures of strain 95-173 suspension containing 9×10^2 cfu to 9×10^7 cfu cells per mL in Phosphate-Buffered Saline (PBS) and 6 normal animals were injected with 0.2 mL PBS as control. All the animals were tested under the same conditions. Mortality was recorded daily for 7 days. The 50% Lethal Doses (LD_{50}) of the bacterium were calculated as described previously (Han *et al.*, 1992).

Type 4 pilli were tested with type 4 pilli probe by clone hybridization. HEC toxin was detected by sheep blood plate. On skim milk plate, proteolytic rings were observed. Bacteria were treated with glycine buffer and then examined under electron microscope; crystal-like S protein was seen. The highest dilution that hemolyzed fifty perecent of human erythrocytes was the hemolytic valence of the strain.

Susceptibilities to 10 different antibiotics (ampicillin, tetracycline, kanamycin, chloramphenicol, furazolidone, erythromycin, gentamycin, oxytetracycline, norfloxacin and trimsulf) and some Chinese herbs (rhubarb, gallnut, forsythia, honeysuckle, plantain, yellow weed and purslane) were tested according to the methods described by Liu (1987).

From Feb. 1995 to July 2003, the unknown disease occurred in three eel farms. In one of them, more than 30 kg of eels died per day and the mortality rate was more than 50%. The diseased eels manifested low appetite, various degrees of bleeding on the head, chest and abdomen and redness and swelling in the anus region. The biopsies showed that a lot of light yellow fluids accumulated in the intestine and ascites and even in stomach of severely diseased eels. And two similar strains were isolated from patients with diarrhoea. The patients were workers on the fish farm where the diseases in eels occurred, the clinical manifests were accute enteritidis symptoms, including diarrhoea, pain in the stomach, nausea and vomitting.

All ten strains were non-spore forming bacilli, Gram-negative, motile, B-hemolytic and produced brown pigments within 24 h of incubation from 25-37°C. The bacteria grew at 37°C in peptone broth without any salt. The biochemical tests demonstrated that the bacteria were members of the genus *Aeromonas* with the following phenotype: glucose fermentation with acid and gas producing, oxidase positive, nitrate reduction positive, O/129 resistant, but they were different from the other members in the genus. The strains were readily distinguished from *A. salmonicida* by their motility and their ability to grow both in broth and on TSBA plate at 37°C. They are also different from brown-pigment-producing *A. media* by their motility and their inability to utilize lactose and arabinose. The major characteristics that differentiate the new species from other *Aeromonas* species are shown in Table 1.

Table 1: Key tests for the phenotypic differentiation of *A. haianensis* from other *Aeromonas* species

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Motility		+	+	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+
Growth at 37°C		+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Brown pigment		-	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	NA
Production of gas from glucose		+	+	+	-	+	+	+	-	-	+	+	+	+	+	+	-	+
Utilization of lactose		-	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-
Decarboxylation of	Ornithine	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
	Arginine	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
	Lysine	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
Production of	Mannitol	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+
acid from	Salicin	-	+	-	+	-	+	+	+	-	-	-	+	-	+	-	+	-
	Sucrose	-	+	-	+	+	+	+	+	-	-	-	-	+	+	-	+	+
Utilization of arabinose		-	-	+	+	-	-	-	+	-	-	-	+	+	-	+	-	-
Hydrolysis of esculin		-	+	+	+	-	+	+	-	-	-	-	+	-	+	-	+	NA
Acetoin production		-	+	-	-	+	+	+	-	-	+	-	-	-	-	+	-	NA

1. A. *enteropelogenes* (J11=DSM 6394), 2. A. *haianensis* (CCTCC AB97026T=IFO 16641), 3. A. *Salmonicida subsp salmonicida* (LMG3780 T=ATCC 33658), 4. A. *caviae* (LMG 3775 T=ATCC 15468), 5. A. *sobria* (LMG 3783 ^{T}=ATCC 43979), 6. *A. hydrophila* (LMG 2844 ^{T}=ATCC 7966), 7. A. *veronii* (LMG 9075 ^{T}=ATCC 35624), 8. *A. media* (LMG 9073 ^{T}=ATCC 33907), 9. *A. shubertii* (LMG 9074 ^{T}=ATCC 43700), 10. *A. jandaei* (LMG 12221 ^{T}=ATCC 49568), 11. *A. trota* (LMG 12223 ^{T}=ATCC 49657), 12. *A. eucrenophila* (LMG 3744 ^{T}), 13. *A. allosaccharophila* (LMG 14059 ^{T}=ATCC 51208), 14. *A. bestiarum* (LMG 13444 ^{T}=ATCC 51108), 15. *A. popoffii* (LMG 17541 ^{T}), 16. *A. encheleia* (LMG 16330 ^{T}=CECT 4343), 17. *A. culicicola*(MTCC3249 ^{T})
Note: symbol "+" stands for positive, "-" for negative. NA, data not available

Table 2: Chromosomall DNA-DNA hybridization results. For DNA-DNA hybridization, the mean±SD is given with the member of replicates in parentheses

	DNA-DNA hybridization %	
Strains	*A. haianensis* 95-72	*A. haianensis* 95-173
A. haianensis 95-72	100	93±2(3)
A. haianensis 95-173=CCTCC AB97026^T=IFO 16641)	94±1(3)	100
A. haianensis 98-15	92±2(3)	89±3(3)
A. hydrophila (LMG 2844^T=ATCC 7966)	59±1(3)	56±2(3)
A.salmonicida salmonicida (LMG 3780^T=ATCC 33658)	58±2(3)	60±3(3)
A. sobria (LMG 3783^T=ATCC 43979)	62±1(3)	61±1(3)
A. trota (LMG 12223^T=ATCC 49657)	62±2(3)	57±2(3)
A. veronii (LMG9075 ^{T}=ATCC 35624)	60±1(3)	63±1(3)
A. caviae (LMG 3775^T=ATCC 15468)	58±2(3)	54±2(3)
A. media (LMG 9073^T=ATCC 33907)	59±2(3)	57±3(3)
A. encheleia (LMG 16330^T=CECT 4342)	59±1(3)	61±1(3)
A. popoffii (LMG 17541^T)	48±3(3)	49±2(3)
A. allosaccharophila (LMG 14059^T=ATCC 51208)	49±2(3)	47±2(3)
A. schubertii (LMG 9074^T=ATCC 43700)	38±4(3)	35±3(3)
A. jandaei (LMG 12221^T=ATCC 49568)	42±2(3)	44±3(3)
A. bestiarum (LMG 13444^T=ATCC 51108)	38±3(3)	41±2(3)
A. eucrenophila (LMG 3774^T)	41±4(3)	45±3(3)

The G+C% content of strain 95-72, 95-173 and 98-15 is 55.8, 55.9 and 56.2%, respectively.

The strains 95-72 and 95-173 showed DNA-DNA hybridization rates (Table 2) less than 70% with the other *Aeromonas* species type strains used.

FAME analysis has been applied to the taxonomic study of *Aeromonas* and has proven to be a valuable taxonomic parameter to characterise *Aeromonas* species (Huys *et al*., 1994, 1995). In this study, we have analyzed CFAs components of strain (95-173) and some reference strains of *Aeromonas* species. The dominating fatty acids in the new isolate are the unsaturated acids, including 16:1ω7c and summed feature 7 (18:1ω7t, 18:1ω9t and /or 18:1ω12t), which account for more than 50 % of the total fatty acids. The 16:0 acids also occupy a significant proportion (up to 13.82%). The data coincide well with other previous results (Huys *et al*., 1995, 1997). It was shown that, possessing abundant unsaturated fatty acids and 16:0 acids is a key feature of aeromonads.

The 16S rRNA gene of strain 95-173 showed 97 to 99% sequence similarity to the 16S rRNA gene of *Aeromonas* species retrieved from DDBJ/EMBL/Genebank database and showed highest similarity to *Aeromonas veronii* with an S-ab value of 0.995. The phylogenetic tree generated by DNAstar software is shown in Fig. 1.

The LD_{50} for mice were detected for strain 95-173, which was 3.9×10^6cfu. Most eels infected with strain 95-72 got sick or died in 3 to 7 days and strain 95-72 could be isolated from liver and intestine of the suffered eels. LD_{50} for these eels was 1.2×10^5cfu.

Strains 95-72, 95-173, 98-15 showed positive results when they hybridized with type 4 pilli probe of *E.coli*. The crystal-like S protein was observed in the electron microscope. On skim milk plate, proteolytic rings were found, indicating all nine strains produced proteinase. All nine strains were β-hemolytic on sheep blood plate and had HEC toxin (Tu and Liu, 1992). The hemolytic valence of the strain was 1:32~ 1:64

In the drug sensitivity test, strains 95-72, 95-173, 96-109, 97-14, 98-15, 03-37 were sensitive to norfloxacin, furazolidine and chinese herbs nutgall, rhubarb, but resistant to penicilin, SMZ, kanamycin, terramycin, gentimycin, tetracycline and Chinese herbs, forsythia, honeysuckle, palntain, yellowweed. MIC of norfloxacin was 0.7 μg mL^{-1}, of nutgall and rhubarb was 12.5 and 0.78 mg mL^{-1}, respectively.

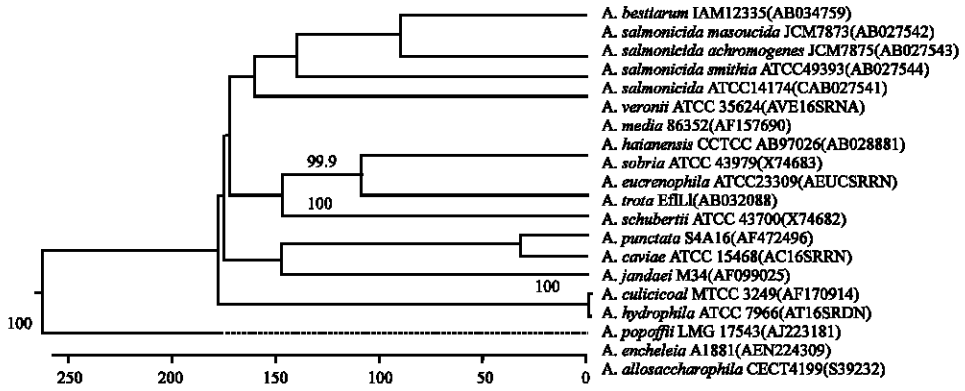

Fig. 1: Unrooted tree showing phylogenetic relationships of *A. haianensis* with other species in genus *Aeromonas*. Bootstrap values less than 50% are not shown. The tree is based on a comparison of 1278 nucleotides (from 8 to 1286) using the neighbor-joining method. (The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/Genbank nucleotide sequence databases with the accession number: AB028881)

According to the results, the ten isolates from the diseased eels' viscera and some patients' stool constitute a separate group within *Aeromonas*, that can be differentiated from the other *Aeromonas* species by phenotypic data, genomic data of strains 95-173; 95-72 and 98-15 (16S rRNA and DNA-DNA hybridization) indicate that these strains constitute a new *Aeromonas* species for which the name *Aeromonas haianensis* is proposed.

Description of *Aeromonas haianensis* sp. nov: *Aeromonas haianensis* (*hai'an'ensis* N. L. fem. adj. *haianensis* of haian, the city in P. R. China where the type strain was isolated). The cells are straight motile rods, which produce a diffusible brown pigment and HEC toxin. They can grow in 0-3% (wv^{-1}) NaCl, but not in 6% NaCl; They produce acid and gas from glucose; lysine decarboxylase and arginine dihydrolase are positive, ornithine decarboxylase is negative, indole and Voges-Proskauer tests are positive. They do not produce H_2S from thiosulphate, they liquefy gelatin and hydrolyse esculin; growth occurs at pH 9.0; but not at pH 4.5; acid is produced from sucrose, D-cellobiose, maltose, D-galactose, D-mannose D-mannitol, but not from adonitol, salicin, D-xylose, lactose, erythritol, dulcitol myo-Insitol, O-sorbitol, L-arabinose, trehalose, melibiose, taffinose and L-rhamnose. The strains can be differentiated from the other *Aeromonas* species (Table 1). The G+C mol% of strains 95-72, 95-173 and 98-15 were 55.8, 55.9 and 56.2% respectively. DNA-NDA hybridization studies were performed to find out the relatedness of strain CCTCC AB97026^T to strain 95-72 and 98-15 and to other *Aeromonas* species (Table 2). Strain CCTCC AB97026^T was found to show the highest DNA-DNA similarity, 63% to *A. veronii* (ATCC 35624) and the lowest value, 35% to *A. schubertii* (ATCC 43700). It showed 61% similarity to *A. sobria* (ATCC 43979) and *A. encheleia* (CECT 4342) and 56% to *A. hydrophila* (ATCC 7966). Strain CCTCC AB97026^T showed 93 and 89% similarity to strain 95-72 and 98-15. The 16S rRNA gene sequence of type strain CCTCC AB97026^T (IFO16641) is highly similar to all known sequences of *Aeromonas veronii* with 6 basic differences.

Aeromonas occurring ubiquitously in dirty water, soil, fish and reptile, have been known as the main pathogen of reptile and amphibians diseases (Lu, 1992). But now it has been shown a new species can also cause a disease in humans. In this report, we isolated indeed *A. haianensis* from viscera of suffered eels. The LD_{50} of these strains for mice is 3.9×10^6 and 1.2×10^5 cfu for eels. Hemolysis valent is 1:32-64, with type 4 pilli, S-layer, proteinase and HEC toxin. All these indicate the strains' strong virulence. Results of antibiotic susceptibility tests show norfloxacin and the Chinese herb nutgall and rhubarb work effectively to cure diseases caused by *A. haianensis*.

The type strain has been deposited at China Center For Type Culture Collection, as strain CCTC AB97026^T and at the Institute For Fermentation, OSAKA, Japan, as strain IFO16641.

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