Aeromonas simiae sp. nov., isolated from monkey faeces

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Two *Aeromonas* strains, IBS S6874^T and IBS S6652, were isolated from the faeces of two healthy monkeys (*Macaca fascicularis*) from Mauritius that were kept in quarantine in the Centre for Primatology, Strasbourg, France. Phylogenetic analysis based on 16S rRNA gene sequences showed that the two isolates formed an unknown genetic lineage within the genus *Aeromonas*. The two isolates had nearly identical sequences (0.1 % nucleotide substitution) that were related closely to those of recognized *Aeromonas* species (1.7–3.5 % nucleotide substitution). DNA–DNA hybridization showed that strains IBS S6874^T and IBS S6652 had high DNA–DNA similarity (89 %) to each other and a low level of DNA–DNA similarity to closely related taxa (18 % relatedness to *Aeromonas trota* and 16 % relatedness to *Aeromonas schubertii*). Phenotypically, the two monkey isolates differed from most previously described mesophilic *Aeromonas* species by their lack of haemolysis on sheep-blood agar and inability to produce indole, gas from glucose or acid from mannitol. They differed from the most closely related species, *A. schubertii*, by their ability to produce acid from D-cellobiose and D-sucrose and by their pyrazinamidase activity. The name *Aeromonas simiae* sp. nov. is proposed for these isolates; strain IBS S6874^T (= CIP 107798^T = CCUG 47378^T) is the type strain.

The taxonomy of the genus *Aeromonas* has undergone continual change, due to reclassification or extended description of existing species and addition of newly described taxa (Huys *et al.*, 1997a, 2002; Pidiyar *et al.*, 2002; Esteve *et al.*, 2003).

Members of the genus *Aeromonas* are widespread in environmental habitats, such as soil (Brandi *et al.*, 1996), fresh and brackish water, sewage and wastewater (Holmes *et al.*, 1996). They are implicated as pathogens of cold-blooded animals and various mammals, including humans, where they cause severe gastroenteritis, soft-tissue infections and bacteraemia (Janda & Abbott, 1998). *Aeromonas* isolates have been recovered from faeces in acute cases of gastroenteritis, as well as in asymptomatic cases (Hänninen & Siitonen, 1995). During a routine survey to determine the presence of possible pathogens in the intestinal tract

of monkeys (*Macaca fascicularis*) from Mauritius that had been put in quarantine in the Centre for Primatology, Louis Pasteur University, Strasbourg, France, two phenotypically related strains, which were identified tentatively as *Aeromonas* sp., were isolated. These strains could not be identified as members of any previously described *Aeromonas* species. In the current investigation, phylogenetic analysis of 16S rRNA gene sequences, DNA–DNA hybridization and extensive phenotypic tests were undertaken to determine the taxonomic position of the two monkey isolates. Based on the reported data, we describe a novel species of the genus *Aeromonas*, for which the name *Aeromonas simiae* sp. nov. is proposed.

Two strains (Institute of Bacteriology of Strasbourg strain IBS S6874^T = CIP 107798^T = CCUG 47378^T and IBS S6652 = CIP 107797) were each isolated from a different sample of faeces from two healthy monkeys (*M. fascicularis*) from Mauritius that were kept in quarantine in the Centre for Primatology, Louis Pasteur University, Strasbourg, France, and were sampled routinely in September 1999. Reference strains of other *Aeromonas* species were obtained from the Collection de l'Institut Pasteur (CIP), Paris, France.

Strains were cultivated aerobically on sheep-blood agar

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Abbreviations: CCUG, Culture Collection, University of Göteborg; CIP, Collection de l'Institut Pasteur.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain IBS S6874^T is AJ536821.

[trypticase soy agar (bioMérieux) that was supplemented with 5 % defibrinated sheep blood] and on Mueller–Hinton agar (Bio-Rad) plates at 30 °C unless indicated otherwise.

Cell shape, Gram-stain and biochemical activities were determined with cultures grown overnight on Mueller–Hinton medium (Smibert & Krieg, 1981). Kovacs cytochrome oxidase was tested by using commercially available discs that were impregnated with dimethylparaphenylene diamine oxalate (Bio-Rad). D-Glucose fermentation by the method of Hugh & Leifson (1953), salt tolerance and resistance to vibriostatic agent O/129 (150 µg) were determined by methods described by Lee & Donovan (1985). Staining of flagella was performed according to the Rhodes method (Buttiaux *et al.*, 1966).

Together with three reference strains of *Aeromonas* (*Aeromonas veronii* CIP 103438^T, *Aeromonas schubertii* CIP 103437^T and *Aeromonas caviae* CIP 76.16^T), they were tested for physiological and biochemical properties according to the identification scheme described by Abbott *et al.* (1992). The following biochemical tests were performed by using previously described methods and incubation was carried out at 30 °C for 48 h: indole production by using peptone water [1 % (w/v) peptone supplemented with 0.5 % tryptophan] (Smibert & Krieg, 1981); gas production from D-glucose (Lee *et al.*, 1981); lysine and ornithine decarboxylase and arginine dihydrolase activities were determined in Fay–Barry medium as described by Altwegg *et al.* (1987). Acetoin production (Voges–Proskauer test) and aesculin hydrolysis were performed as described by Lee & Donovan (1985). Fermentation of carbohydrates (acid production) was determined in nutrient broth (Difco) that was supplemented with 0.002 % phenol red and one of the following substrates: lactose, D-mannitol, sucrose, L-arabinose, salicin or D-cellobiose. Yellow colour of the liquid medium was considered to be a positive reaction. Haemolysis of sheep blood was determined on trypticase soy agar supplemented with 5 % defibrinated sheep blood. Pyrazinamidase activity was determined by using pyrazinamidase diagnostic tablets (Eurobio Rosco) according to the manufacturer's instructions. For additional biochemical tests, API 50CH strips (bioMérieux) were used according to the manufacturer's instructions. Growth at 37 and 42 °C was tested in nutrient broth (Difco); cultures were incubated for 24 h. Susceptibility to cephalothin and ampicillin was tested by the Bauer–Kirby disc diffusion method (Bauer *et al.*, 1966).

For small-subunit rDNA sequencing and phylogenetic study, strains were cultured on Columbia agar (Difco) at 37 °C. DNA was extracted and purified with an IsoQuick Nucleic Acid Extraction kit (ORCA Research). Using universal primers, 1.5 kb of the 16S rRNA gene was amplified by PCR: the primers used were A, corresponding to positions 8–28 of the *Escherichia coli* 16S rRNA gene (Edwards *et al.*, 1989), and rJ, complementary to positions 1510–1492 (Janvier & Grimont, 1995).

The amplification reaction was carried out in a final volume

of 100 µl, with 1 µl template, 20 pmol each primer ml⁻¹, 200 µM each dNTP, 50 µM MgCl₂, 2.5 U *Taq* polymerase and 10 µl 10 × reaction buffer (Amersham Biosciences). Initial denaturation was carried out for 4 min at 94 °C. Amplification (35 cycles) was performed in a DNA Thermal Cycler 9700 (PE Applied Biosystems). Each cycle consisted of three steps: denaturation at 94 °C for 1 min, annealing at 49 °C for 1 min and extension at 72 °C for 2 min. A final extension was performed at 72 °C for 5 min.

PCR products were detected by electrophoresis on 0.8 % agarose gels in Tris/borate/EDTA buffer (0.089 M Tris/borate, 0.018 M EDTA) with 1 kb DNA ladder (Gibco-BRL) as the molecular size marker.

The amplified product was sequenced by Genome Express (Meylan, France) with three primers, E, rE and D, which are located in conserved regions of the *E. coli* 16S rRNA gene. Primer E corresponded to positions 787–806, primer rE was complementary to primer E and primer D corresponded to positions 519–536 [numbering according to Brosius *et al.* (1978)].

Sequences of strains IBS S6874^T (GenBank accession no. AJ536821) and IBS S6652 (AJ536820) were compared and aligned with those of other strains, which were obtained from GenBank (<http://www.ncbi.nlm.nih.gov/>) by using BLAST (NCBI) and the MegAlign module of the Lasergene software (DNASTAR). A phylogenetic tree was generated by using the neighbour-joining algorithm (Saitou & Nei, 1987).

DNA–DNA hybridization was performed as described previously (Riegel *et al.*, 1994). Briefly, DNA was extracted from SDS-treated cells of strains IBS S6874^T, IBS S6652, *A. schubertii* CIP 103437^T and *Aeromonas trota* CIP 103677^T by using a phenol/chloroform-based procedure. DNA was quantified spectrophotometrically and the purity of each preparation was ascertained by measuring the *A*₂₈₀/*A*₂₆₀ and *A*₂₃₀/*A*₂₆₀ ratios. Hybridization between labelled DNA and the fragmented DNA preparation was carried out at 60 °C for 16 h in 0.42 M NaCl by the nuclease/trichloroacetic acid method (Grimont *et al.*, 1980). Values of DNA–DNA relatedness that are given are means of the results of three independent experiments.

Isolates IBS S6874^T and IBS S6652, which are studied herein, were recovered on blood agar plates from different faeces samples from two healthy monkeys. These two isolates were identified as members of the genus *Aeromonas*, as they were Gram-negative rods with polar flagella that were oxidase-positive, glucose-fermenting, facultatively anaerobic, unable to grow in nutrient broth with 6 % NaCl and resistant to vibriostatic agent O/129 (150 µg). The biochemical patterns of these two strains were nearly identical and did not permit their assignment to any recognized *Aeromonas* species. In addition, there was 89 % DNA–DNA hybridization between isolates IBS S6874^T and IBS S6652, indicating that these two strains should be assigned to the same genomospecies.

In order to determine the taxonomic position of these two strains, which differ from recognized *Aeromonas* species, we studied them and reference strains by performing DNA–DNA hybridization analysis and small-subunit rDNA sequence analysis. The almost-complete sequence of strain IBS S6874^T (1452 bp), and a large part of the sequence of strain IBS S6652 (1239 bp), were determined. Phylogenetic analyses confirmed that the two strains belonged to the robust monophyletic unit that groups all available *Aeromonas* sequences together (Fig. 1). Comparison of 16S rDNA sequences revealed that those from the two strains exhibited a high level of similarity to each other (0.1 % nucleotide substitution), but that they represented an unknown phylogenetic lineage within the genus *Aeromonas*. The lowest level of nucleotide substitution between strain IBS S6874^T and a currently recognized *Aeromonas* species was 1.7 % (*A. schubertii*); nucleotide substitution with other representatives of the genus *Aeromonas* (listed in Fig. 1) ranged from 2.0 to 3.5 %. It is noteworthy that these values are above the nucleotide substitution values that are usually obtained between recognized species of the genus *Aeromonas* (Martínez-Murcia *et al.*, 1992a, b; Pidiyar *et al.*, 2002). According to the limitations of 16S rDNA analysis in the determination of novel taxa (Martínez-Murcia, 1999), DNA–DNA

hybridization was performed between strain IBS S6874^T and reference strains *A. trota* CIP 103677^T and *A. schubertii* CIP 103437^T, yielding values of 18 and 16 %, respectively. Thus, DNA–DNA hybridization clearly segregated strain IBS S6874^T from *A. trota* and *A. schubertii*, which were closely related taxa on the basis of 16S rDNA sequence comparison analysis. Therefore, according to the level of phylogenetic diversity within the genus *Aeromonas* and following the criteria proposed by Wayne *et al.* (1987) and Stackebrandt & Goebel (1994), our 16S rDNA sequence and DNA–DNA hybridization data clearly show that the two monkey isolates form a novel genomic species within the genus *Aeromonas*.

Similar results were obtained by classical methods of carbohydrate fermentation determined on nutrient broth and by API 50CH strips (data not shown). Table 1 shows selected biochemical characters for phenotypic differentiation from other *Aeromonas* species. Thus, the two isolates differ from most previously described mesophilic *Aeromonas* species, except for *A. schubertii*, by their inability to produce indole, gas from glucose and acid from D-mannitol. The monkey isolates differ from *A. schubertii* by their ability to produce acid from D-cellobiose and D-sucrose and by their pyrazinamidase activity. The two isolates could be

Fig. 1. Phylogenetic tree of 16S rRNA gene sequences of strains IBS S6874^T, IBS S6652, representative *Aeromonas* species and some relatives, constructed by using the neighbour-joining method (Saitou & Nei, 1987). GenBank accession numbers are given in parentheses.

Table 1. Key tests for phenotypic differentiation of *Aeromonas simiae* from other *Aeromonas* species

Taxa are identified as: 1, *A. simiae* sp. nov.; 2, *Aeromonas hydrophila*; 3, *A. hydrophila* subsp. *dhakensis*; 4, *Aeromonas bestiarum*; 5, *A. caviae*; 6, *Aeromonas media*; 7, *Aeromonas eucrenophila*; 8, *Aeromonas sobria*; 9, *A. veronii* biogroup *sobria*; 10, *Aeromonas culicicola*; 11, *A. veronii* biogroup *veronii*; 12, *Aeromonas jandaei*; 13, *Aeromonas encheleia*; 14, *A. schubertii*; 15, *A. trota*; 16, *Aeromonas allosaccharophila*; 17, *Aeromonas popoffii*. Data were taken from Abbott *et al.* (1992, 2003), Martínez-Murcia *et al.* (1992b), Esteve *et al.* (1995, 2003), Ali *et al.* (1996), Huys *et al.* (1997b, 2002), Altwegg (1999) and Pidiyar *et al.* (2002). Characters are scored as: +, >85 % of positive strains; –, <15 % of positive strains; V+, 60–85 % of positive strains; V–, 15–40 % of positive strains; V, around 50 % of positive strains; ND, no data from these authors.

Character	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Production of gas from glucose	–	+	ND	V+	–	–	+	V	+	+	V+	+	V+	–	V+	+	+
Hydrolysis of aesculin	V	+	+	+	+	+	+	–	–	ND	+	–	+	–	–	V+	–
Indole production	–	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	V+
Acid from:																	
Salicin	–	+	+	V+	+	V–	+	–	–	–	+	–	+	–	–	–	–
D-Cellobiose	+	–	–	–	+	+	+	+	V+	–	V+	–	–	–	+	+	–
D-Mannitol	–	+	+	+	+	+	+	+	+	+	+	+	+	–	V+	+	+
D-Sucrose	+	+	+	+	+	+	V+	+	+	+	+	V+	V+	–	V–	+	–
L-Arabinose	–	+	–	+	+	+	V+	–	V–	–	–	–	–	–	–	V+	V
Decarboxylation of:																	
Ornithine	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	V–	–
Lysine	+	+	+	+	–	–	–	+	+	+	+	+	–	V+	+	+	–
Arginine dihydrolase	+	+	+	+	+	+	+	–	+	+	–	+	+	+	+	V+	+
Pyrazinamidase activity	+	+	ND	V+	V+	V–	+	–	–	ND	–	–	–	–	–	V–	–

differentiated from one another by some phenotypic features: according to the data obtained by using API 50CH strips, strain IBS S6874^T produced acid from arbutin, but did not hydrolyse aesculin after 48 h incubation, in contrast to strain IBS S6652.

As strains IBS S6874^T and IBS S6652 constitute a distinct genomic species that can be identified by phenotypic properties, they represent a novel species, for which the name *Aeromonas simiae* sp. nov. is proposed.

Description of *Aeromonas simiae* sp. nov.

Aeromonas simiae (si'mi.ae. Gr. n. *simia* monkey; N.L. adj. *simiae* from monkeys).

Strains display the following characteristics that are typical of the genus *Aeromonas*: Gram-negative, straight, motile rods with a polar flagellum, cytochrome oxidase-positive, chemoorganotrophic with both oxidative and fermentative metabolisms, catalase-positive, reduce nitrate to nitrite without production of gas and resistant to vibriostatic agent O/129 (150 µg). Unable to grow in nutrient broth with 6 % NaCl. Optimal growth occurs at 30 °C after 24 h on trypticase soy agar or sheep-blood agar, but strains also grow at 42 °C after 24 h. Unable to produce indole, gas from glucose or β -haemolysis on sheep-blood agar. Voges–Proskauer- and ornithine decarboxylase-negative; lysine decarboxylase-, arginine dihydrolase- and pyrazinamidase-positive. Hydrolysis of aesculin and acid production from arbutin are variable. By fermentation, acid is produced

from glycerol, ribose, galactose, D-glucose, D-fructose, D-mannose, N-acetylglucosamine, D-cellobiose, D-sucrose, D-maltose, D-trehalose, starch, glycogen and gluconate, but not from erythritol, D-arabinose, L-arabinose, D-xylose, L-xylose, adonitol, methyl β -D-xyloside, sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, methyl α -D-mannoside, methyl α -D-glucoside, amygdalin, melibiose, inulin, melezitose, raffinose, xylitol, gentiobiose, turanose, D-lyxose, D-tagatose, D-fucose, D-arabitol, 2-ketogluconate, 5-ketogluconate, salicin or lactose. Major characteristics that differentiate the species from other *Aeromonas* species are shown in Table 1. Susceptible to cephalothin, but resistant to ampicillin.

The type strain, IBS S6874^T (=CIP 107798^T=CCUG 47378^T), was isolated from the faeces of a monkey (*Macaca fascicularis*) that was kept in the Centre for Primatology, Louis Pasteur University, Strasbourg, France. This strain has all of the above-described properties for the species; in addition, aesculin is not hydrolysed after 48 h incubation and acid is produced from arbutin.

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