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1 **Phylogenetic identification of *Aeromonas simiae* from a pig, first isolate since species**  
2 **description**

3

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6

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10

11 **Abstract**

12 The species *Aeromonas simiae* was first described on the basis of two strains (CIP  
13 107798<sup>T</sup> and CIP 107797) isolated from faeces of healthy monkeys (*M. fascicularis*) from  
14 Mauritius, which were kept in quarantine in the Centre for Primatology, Louis Pasteur University,  
15 Strasbourg, France. In the present study, during a survey to determine the prevalence of  
16 *Aeromonas* at three different pig slaughterhouses at north of Portugal, a single strain MDC2374  
17 (out of 703 isolates) was identified as *Aeromonas simiae* on the basis of 16S rDNA, *gyrB* and  
18 *rpoD* sequencing, confirming that traditional biochemical approaches are not resolute to identify  
19 rarely isolated *Aeromonas* species. To our knowledge, this is the first time since species  
20 description that *A. simiae* is newly isolated and identified. .

21

22 **Keywords:** *Aeromonas simiae*; phylogeny; 16S rDNA; *gyrB*; *rpoD*.

23

24

## 25 1. Introduction

26 The taxonomy of the genus *Aeromonas* is complex and continually changes due to the addition of  
27 newly described species and the reclassification or extended description of existing taxa. The  
28 genus *Aeromonas* belongs to the family *Aeromonadaceae* (Colwell *et al.*, 1986) and its members  
29 are gram-negative, non-spore-forming bacilli or coccobacilli, oxidase and catalase-positive,  
30 facultatively anaerobic, resistant to the vibriostatic agent O/129 and generally motile by a polar  
31 flagella (Martin-Carnahan & Joseph, 2005). Members of the genus *Aeromonas* are human  
32 pathogens, being associated with a wide spectrum of enteric and non-enteric diseases in (Janda  
33 & Abbott, 1998). *Aeromonas* species are common inhabitants of aquatic ecosystems. They have  
34 also been isolated in different kinds of animals and food (Isonhood & Drake, 2002). Furthermore,  
35 *Aeromonas* spp. have been isolated from pig carcasses and faeces and also from processing  
36 equipment (Gill and Jones 1995; Saavedra *et al.*, 2007).

37 *Aeromonas simiae* was described on the basis of two strains (CIP 107798<sup>T</sup> e CIP 107797)  
38 isolated from faeces of healthy monkeys (*M. fascicularis*) from Mauritius, that were kept in  
39 quarantine in the Centre for Primatology, Louis Pasteur University, Strasbourg, France. To our  
40 knowledge, since its description (Harf-Monteil *et al.*, 2004), no more isolates of this species have  
41 been identified. Further studies showed identical *gyrB*, *rpoD* and 16S rDNA sequences (Saavedra  
42 *et al.*, 2006) and the same genotyping pattern (Figueras *et al.*, 2006), revealing that they are two  
43 isolates of a single strain. The report of the ad-hoc committee for the re-evaluation of the species  
44 definition in bacteriology encourages microbiologists to base a species description on more than  
45 a single strain, recommends the analysis of housekeeping gene sequences and the application of  
46 DNA profiling methods (Stackebrandt *et al.*, 2002). Sequencing of *gyrB* and *rpoD* has  
47 demonstrated that both genes are excellent molecular chronometers for phylogenetic inference in  
48 the genus *Aeromonas* (Yañez *et al.*, 2003; Soler *et al.*, 2004; Saavedra *et al.*, 2006; Küpfer *et al.*,  
49 2006). In the present study, based on *gyrB*, *rpoD* and 16s rDNA gene sequencing, we report the

50 phylogenetic identification of one strain of *A. simiae* (MDC2374) from pig faeces, isolated in a  
51 slaughterhouse in Portugal.

52

## 53 **2. Materials and Methods**

### 54 **2.1. Sample collection and isolation of *Aeromonas* species**

55 A total of 134 samples (44 pig carcasses intended for human consumption, 25 diaphragm  
56 muscle, 66 pig faeces, 6 samples of processing equipment and 12 samples of water) were  
57 obtained from three different pig slaughterhouses and kept at 4°C until laboratory examination.  
58 The 66 samples of pig faeces consisted in small portions of ileum with faeces. At the laboratory  
59 25 g of ileum with faeces (after being scalded) were weighted aseptically, homogenised for at  
60 least 2 minutes in stomacher bags containing 225 ml of alkaline peptone and incubated 24 h at  
61 30°C. After enrichment, the homogenate was streaked on glutamate starch phenol-red agar  
62 (GSP) agar (Merck) plates. After incubation at 30°C for 24h, the yellow colonies were isolated  
63 and subcultured into tryptone soya agar (TSA) (Oxoid). Oxidase positive colonies were taken as  
64 presumptive *Aeromonas* isolates. Culture collection strains CIP 107798<sup>T</sup> and CIP 107797  
65 (MDC55) were purchased from the Spanish Collection of Type Cultures (CECT). Strains were  
66 maintained on TSA (Oxoid) plates at 4°C and were stored in 10 % glycerol at -20°C.

67

### 68 **2.2. Biochemical Characterization**

69 The biochemical tests for the strain MDC2374 were determined after overnight growth on  
70 TSA plates (Oxoid) and performed using the commercial Kits API 20 E and API 20 NE  
71 (BioMérieux) and the automated Vitek system (BioMérieux). The fermentation (acid production) of  
72 D-mannitol, L-arabinose, D-cellobiose, D-sucrose and salicin was determined in Andrade peptone  
73 water (Sigma). The pink colour of the medium was considered to be a positive reaction.

74

### 75 **2.3. DNA extraction and Enterobacterial Repetitive Intergenic Consensus-PCR (ERIC-PCR )**

76 DNA extraction was performed by resuspending a single colony from fresh cultures in  
77 100 µl of TE buffer and 200 µl of Chelex (BioRad) were added. Tube were vortexed at high  
78 speed for 1 min, incubated at 96°C for 10 min and kept at -20°C for 10 min. This process was  
79 repeated three consecutive times. The tube was again vortexed and centrifuged for 5 min at 12  
80 000 g. The supernatant was transferred to a fresh tube and stored at -20°C.

81 All isolates from the same sample were subjected to ERIC-PCR in order to recognize  
82 identical strains. Isolates showing different ERIC-PCR patterns were subjected to further  
83 phylogenetic analysis. The oligonucleotides primers used for ERIC-PCR were ERIC 1R (5' -ATG  
84 TAA GCT CCT GGG GAT TCA C- 3') and ERIC 2 (5' -AAG TAA GTG ACT GGG GTG AGC G-  
85 3') (Versalovic et al., 1991). PCR amplifications were performed in a total volume of 25 µl,  
86 containing 2,5 µl of 10x PCR buffer, 50 pmol of each primer, 400 µM of dNTP's, 1 µl (1U) of *taq*  
87 DNA polymerase and 2,5 µl (50 ng) of template DNA. The reaction mixtures were subjected, on a  
88 MJ Research PTC100 thermal cycler, to a initial denaturation for 4 min at 94 °C followed by 35  
89 cycles, with a thermal cycling of 94 °C for 15 s, 55 °C for 30 s, 72 °C for 45 s and a single final  
90 extension for 5 min at 72 °C. Following amplification, 8 µl of the PCR products were  
91 electrophoresed on 1.2 % agarose gels in TAE buffer. The 1 kb DNA ladder was used as  
92 molecular size marker. Agarose gels were stained on ethidium bromide, visualized with an  
93 ultraviolet lamp and photographed in the GelDocMega cabinet.

94

### 95 **2.4. PCR amplification and sequencing of *gyrB*, *rpoD*, and 16S rDNA**

96 PCR amplification and procedures for sequencing of *gyrB*, *rpoD* and 16S rRNA genes  
97 were performed at the Molecular Diagnostics Center (MDC), as previous described (Martínez-  
98 Murcia *et al.* 1999; Soler *et al.* 2004), except for using the BigDye Terminator V3.1 Cycle

99 Sequencing Kit in the ABI 3100-Avant Genetic Analyzer (Applied Biosystems), according to the  
100 manufacturer's instructions.

101

## 102 **2.5. Phylogenetic data analysis**

103 The nucleotide sequences were aligned by the Clustal X program version 1.8 (Thompson  
104 *et al.* 1997). For alignments, previously published reference sequences (Martinez-Murcia *et al.*  
105 1992a; Martínez-Murcia 1999; Yañez *et al.*, 2003; Soler *et al.*, 2004; Saavedra *et al.*, 2006 ) were  
106 used. Genetic distances were obtained by Kimura's 2 parameter model (Kimura 1980) and  
107 evolutionary tree was constructed by the Neighbour-Joining method (Saitou and Nei 1987) with  
108 the Mega program (Kumar *et al.*, 2001).

109

## 110 **3. Results and discussion**

111 In this survey, *Aeromonas* spp. was detected in 104 out of 134 samples collected in the pig  
112 slaughterhouses. A total of 480 *Aeromonas* were isolated from pig carcasses, diaphragm muscle,  
113 dehairing equipment and water, but also 223 *Aeromonas* isolates were obtained from pig faeces  
114 (data not shown). To avoid redundancy, isolates were subjected to ERIC-PCR analysis and 66  
115 strains, showing different genetic patterns from each other, were selected for phylogenetic  
116 classification by *gyrB* gene sequencing. The obtained *gyrB* phylogenetic analysis showed that  
117 strain MDC2374 clustered with the type strain of the species *Aeromonas simiae*. For this strain,  
118 nucleotide sequences of 16S rRNA, *rpoD* amplicons were also obtained. Experiments were  
119 repeated at least twice from single colonies of original cultures to confirm readings and to solve  
120 ambiguities. The derived *rpoD* sequences were concatenated with these of *gyrB*, aligned with  
121 sequences from reference strains of all *Aeromonas* species described to date, including the  
122 culture collection strains CIP 107798<sup>T</sup> and CIP 107797, and a phylogenetic tree was constructed  
123 (Fig. 1). The *gyrB* and *rpoD* sequences obtained (GenBank accession numbers GQ860942 and

124 GQ860943, respectively) from this isolate showed seventeen and two differences, respectively,  
125 with these of *A. simiae* reference strains (CIP 107798<sup>T</sup> and CIP 107797), This result confirmed  
126 that isolated strain MDC2374 represents a new member of the species *Aeromonas simiae*..

127 The strain obtained in this study and the culture collection strains CIP 107798<sup>T</sup> and CIP  
128 107797 were also subjected to 16S rDNA sequencing (approx. 1400 bp) to check previously  
129 reported data and derived phylogenetic 16S rDNA tree (Fig. 2) showed this strain belongs to *A.*  
130 *simiae*. Sequence of strain MDC2374 (GenBank accession number GQ860944) presented a  
131 variation at 9 nucleotide positions with *A. simiae* reference strains (Table 1). The 16S rDNA  
132 sequences from strains CIP 107798<sup>T</sup> and CIP 107797 were identical (GenBank accession  
133 number GQ860945), supporting data previously published (Saavedra et al., 2006).

134 Biochemically, MDC2374 differs from previously described mesophilic *Aeromonas*  
135 species, except for *A. schubertii*, by its inability to produce indole, gas from glucose and acid from  
136 D- mannitol, in agreement to Harf-Monteil and collaborators (2004) respect to type strain CIP  
137 107798<sup>T</sup> . Moreover, strain MDC2374 may be differentiated from *A. schubertii* by its ability to  
138 produce acid from D-cellobiose and D-sucrose, butalso its inability to produce acetoin from  
139 sodium pyruvate (Voges Proskauer test negative).

140 To our knowledge, it is the first time that *A. simiae* is newly identified since first  
141 description. The results demonstrate that housekeeping gene sequencing is invaluable in the  
142 finding of strains belonging to rarely isolated *Aeromonas* species such as *A. simiae*, and  
143 confirmed its potential as a rigorous frame for *Aeromonas* taxonomy. The finding of a new *A.*  
144 *simiae* strains would allow establishing a phylogenetic cluster of the species *A. simiae* more  
145 diverse and robust.

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159

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223 **Figure Legends**

224 Figure 1:

225 **Phylogenetic tree showing the relationships of strains of *Aeromonas simiae* and all other**  
226 **known species based on the *gyrB-rpoD* gene sequences. Numbers shown next to each**  
227 **node indicate bootstrap values (percentages of 1000 replicates).**

228 Figure 2:

229 **Phylogenetic tree showing the relationships of strains of *Aeromonas simiae* and all other**  
230 **known species based on the 16S rDNA sequences. Numbers shown next to each node**  
231 **indicate bootstrap values (percentages of 1000 replicates).**

Fig. 1

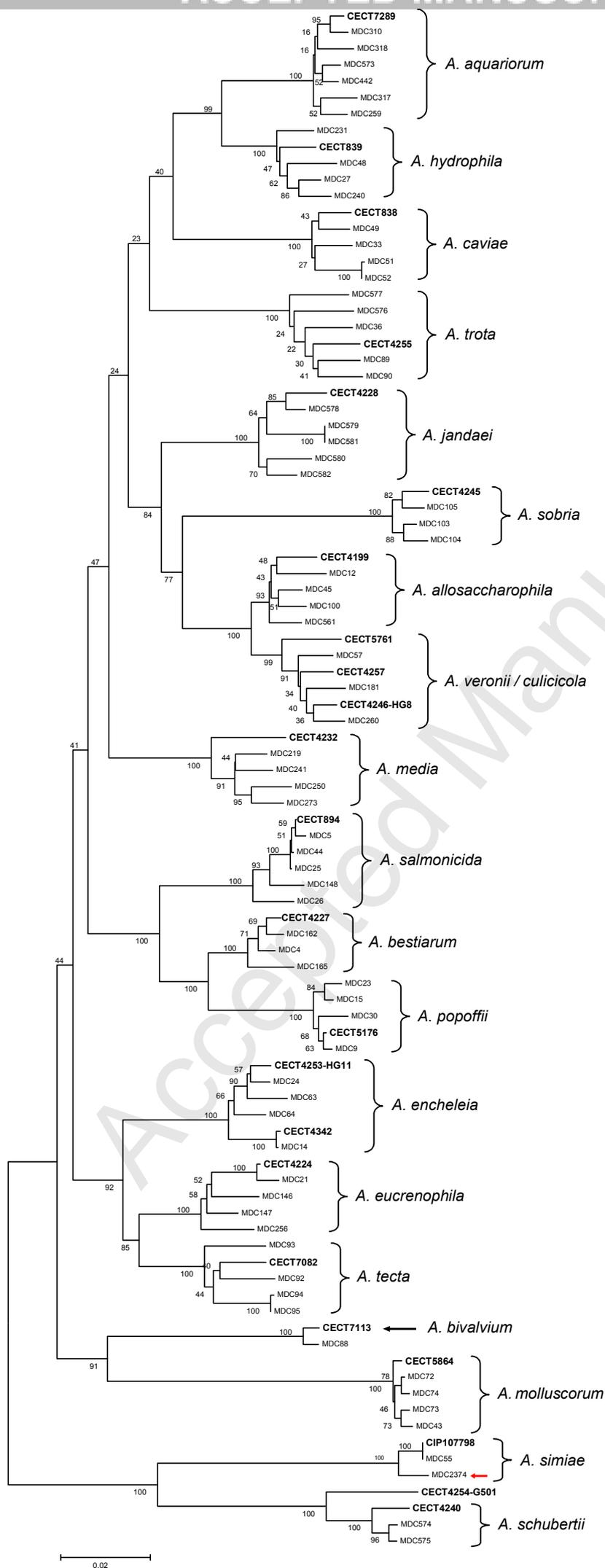


Fig. 2

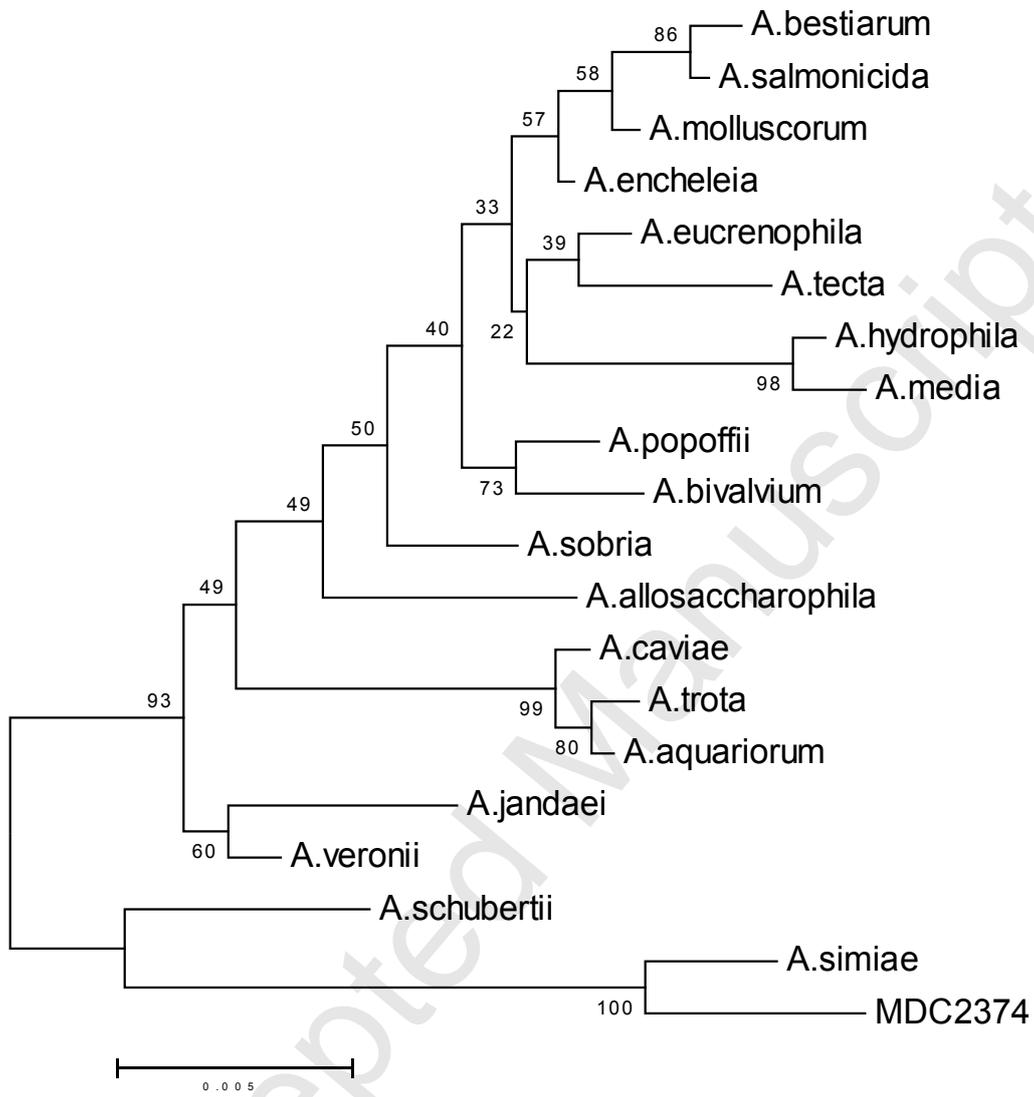


Table 1. Nucleotide differences in the 16S rDNA gene sequences of *Aeromonas simiae* strains.

Strain	Positions at 16S rDNA*									
	250	456	459	461	469	471	472	473	476	647
<sup>1</sup> CIP 107798 <sup>T</sup>	T	T	G	A	A	T	G	C	G	C
MDC2374	A	G	T	G	T	C	A	G	T	T

\* Following the *E.coli* numbering system. <sup>1</sup>Strain CIP 107797 showed identical sequence.

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