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

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ABSTRACT

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

1. Introduction

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

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

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gene sequencing, we report the phylogenetic identification of one strain of *A. simiae* (MDC2374) from pig faeces, isolated in a slaughterhouse in Portugal.

2. Materials and methods

2.1. Sample collection and isolation of Aeromonas species

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

2.2. Biochemical characterization

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

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

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

All isolates from the same sample were subjected to ERIC-PCR in order to recognize identical strains. Isolates showing different ERIC-PCR patterns were subjected to further phylogenetic analysis. The oligonucleotide primers used for ERIC-PCR were ERIC 1R (5′-ATG TAA GCT CCT GGG GAT TCA C-3′) and ERIC 2 (5′-AAG TAA GTG ACT GGG GTG AGC G-3′) (Versalovic et al., 1991). PCR amplifications were performed in a total volume of 25 μl, containing 2.5 μl of 10× PCR buffer, 50 pmol of each primer, 400 μM of dNTPs, 1 μl (1 U) of taq DNA polymerase and 2.5 μl (50 ng) of template DNA. The reaction mixtures were subjected, on a MJ Research PTC100 thermal cycler, to a initial denaturation for 4 min at 94 °C followed by 35 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 extension for 5 min at 72 °C. Following amplification, 8 μl of the PCR products was electrophoresed on 1.2% agarose gels in TAE buffer. The 1 kb DNA ladder was used as molecular size marker. Agarose gels were stained on ethidium bromide, visualized with an ultraviolet lamp and photographed in the GelDocMega cabinet.

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

PCR amplification and procedures for sequencing *gyrB*, *rpoD* and 16S rRNA genes were performed at the Molecular Diagnostics Center (MDC), as previous described (Martínez-Murcia et al., 1999; Soler et al., 2004), except for using the BigDye Terminator V3.1 Cycle Sequencing Kit in the ABI 3100-Avant Genetic Analyzer (Applied Biosystems), according to the manufacturer's instructions.

2.5. Phylogenetic data analysis

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

3. Results and discussion

In this survey, *Aeromonas* spp. was detected in 104 out of 134 samples collected in the pig slaughterhouses. A total of 480 *Aeromonas* were isolated from pig carcasses, diaphragm muscle, dehairing equipment and water, but also 223 *Aeromonas* isolates were obtained from pig faeces (data not shown). To avoid redundancy, isolates were subjected to ERIC-PCR analysis and 66 strains, showing different genetic patterns from each other, were selected for phylogenetic classification by *gyrB* gene sequencing. The obtained gyrB phylogenetic analysis showed that strain MDC2374 clustered with the type strain of the species *A. simiae*. For this strain, nucleotide sequences of 16S rRNA, *rpo*D amplicons were also obtained. Experiments were repeated at least twice from single colonies of original cultures to confirm readings and to solve ambiguities. The derived *rpo*D sequences were concatenated with these of gyrB, aligned with sequences from reference strains of all *Aeromonas* species described to date, including the culture collection strains CIP 107798^T and CIP 107797, and a phylogenetic tree was constructed (Fig. 1). The *gyrB* and *rpoD* sequences obtained (GenBank accession numbers GQ860942 and GQ860943, respectively) from this isolate showed 17 and 2 differences,