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Comparison of phenotypical and genetic identification of *Aeromonas* strains isolated from diseased fish

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ABSTRACT

Phenotypicaly identified *Aeromonas* strains (n=119) recovered mainly from diseased fish were genetically re-identified and the concordance between the results was analysed. Molecular characterization based on the GCAT genus specific gene showed that only 90 (75.6%) strains belonged to the genus *Aeromonas*. The 16S rDNA-RFLP method identified correctly most of the strains with the exception of a few that belonged to *A. bestiarum*, *A. salmonicida* or *A. piscicola. Separation of these* 3 species was correctly assessed with the *rpoD* gene sequences, which revealed that 5 strains with the RFLP pattern of *A. salmonicida* belonged to *A. piscicola,* as did 1 strain with the pattern of *A. bestiarum*. Correct phenotypic identification occurred in only 32 (35.5%) of the 90 strains. Only 14 (21.8%) of the 64 phenotypically identified *A. hydrophila* strains belonged to this species. However, coincident results were obtained in 88% (15/17) of the genetically identified *A. salmonicida* strains. Phenotypic tests were re-evaluated on the 90 genetically characterized *Aeromonas* strains and there were contradictions in the species *A. sobria* for a number of previously published species-specific traits. After genetic identification, the prevailing species were *A. sobria*, *A. salmonicida*, *A. bestiarum*, *A. hydrophila*, *A. piscicola* and *A. media* but we could also identify a new isolate of the recently described species *A. tecta*. This work emphasizes the need to rely on the 16S rDNA-RFLP method and sequencing of housekeeping genes such as *rpoD* for the correct identification of *Aeromonas* strains.

Introduction

The genus *Aeromonas* comprises bacteria that are autochthonous of aquatic environments worldwide and have been implicated in a wide spectrum of diseases in both humans and fish [4,12,21,26]. Species, such as *A. hydrophila*, *A. bestiarum*, *A. veronii* biovar *sobria* and *A. sobria* have been associated with infections in various fish species [23,36]. However, the species *A. salmonicida* has been described as the cause of massive mortality and great economic loss in marine and continental aquaculture, particularly affecting salmonids [5,16,19,31]. The species is divided into four psycrophilic subspecies and one mesophilic subspecies [26,34]. Other motile *A. salmonicida* mesophilic strains have been described and are commonly misidentified as *A. bestiarum* or *A. hydrophila* [9,24,28]. Two studies have proved that genetic techniques based on the sequences of the 16S rRNA gene and DNA–DNA hybridization were not discriminatory for *A. bestiarum* and *A. salmonicida* strains [27,28]. However, these two species could be differentiated on the basis of the *rpoD* and *gyrB* genes [39]. The taxonomy of the genus *Aeromonas* has changed over the last two decades with the addition of an increasing number of species [3,6,11 and references therein]. One major difficulty in the phenotypic identification of *Aeromonas* spp. is the variety of conditions and methodologies employed for the biochemical tests that produce different and/or inconsistent results [1,26,29,41]. The use of molecular approaches has led to a more refined identification of *Aeromonas* spp. and has highlighted considerable number of discrepancies in biochemical identification with both environmental and clinical isolates [7,9,15,24,32,33,38]. A previously published protocol based on RFLP of the 16S rRNA gene has enabled identification of all *Aeromonas* species described up to the year 2000 [7,14]. However, some strains (8%) have produced unexpected or atypical restriction patterns making their identification uncertain, although they were correctly identified using housekeeping gene (*gyrB, rpoD*) sequences [2,28]. Therefore, a combination of 16S rDNA-RFLP analysis [7,14] and sequencing of the gene *rpoD* [2,13,28,39,40] is a suitable approach for the correct assignation of *Aeromonas* strains to recognized species. Despite the 16S rDNA-RFLP protocol [7,14] having been used to analyse the diversity of *Aeromonas* species in frozen fish [9] and in healthy and diseased common carp [23,24], no studies in relation to fish disease have used housekeeping genes for identification purposes.

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The only exception is a very recent study that investigated the strains involved in three outbreaks affecting the Artic char (*Salvelinus alpinus*) [16].

In the present work 119 strains, mainly isolated from diseased fish and phenotypically classified as *Aeromonas* were genetically re-identified (16S rDNA-RFLP and sequencing of the gene *rpoD*). The degree of concordance between these methods was analysed. Additionally, biochemical tests were performed on the genetically identified *Aeromonas* strains and compared to previously published data.

Material and methods

Bacterial isolates and phenotypic identification

Presumptive *Aeromonas* strains (n=119) were isolated from diseased fish from a variety of fish farms, except for two strains isolated from cultured clams (*Ruditapes philippinarum*) and one strain isolated from a king prawn (*Penaeus kerathurus*). Table S1 details the origins and dates of strain isolation. Bacteria were grown on plates of tryptic soy agar supplemented with NaCl to a final concentration of 1% (TSA-1, Pronadisa, Spain) at 25 ± 1 °C for 24 h. The isolates were presumptively identified as *Aeromonas* species by the following classical phenotypic tests: Gram-staining, cytochrome oxidase, catalase, nitrate reduction, motility, fermentation of glucose, indole production, Voges–Proskauer (VP), gas production from D-glucose, hydrolysis of gelatin, lipase (Tween 80) and esculin. Decarboxylation of lysine and ornitine (LDC, ODC) and hydrolysis of arginine dihydrolase (ADH) were evaluated using the Moeller's method however, ADH was carried out using the Thornley's method as well. Resistance to 2,4-diamino-6,7-diisopropylpteridine (vibriostatic agent 0/129; 150 μg), ability to grow at 0% and 6% NaCl and the use of inositol were also tested.

Genetic identification

DNA extraction, amplification primers and conditions for the glycerophospholipid-cholesterol acyltransferase (GCAT) (237 bp), the 16S rRNA gene (1503 bp) as well as those for sequencing and phylogenetic analysis of the *rpoD* gene (820 bp) were described previously [2,10,27,39]. Digestion and analysis of the 16S rRNA gene PCR products were carried out according to previous studies [7,14].

Re-evaluation of phenotypic tests

Thirty-two biochemical tests, including all previously mentioned ones and others listed in Table 2 were performed on the genetically identified *Aeromonas* strains [8,37] at 30 °C except for *A. salmonicida* strains, which were incubated at 25 ± 1 °C. The tests were also performed in parallel for the type strains (Table S2).

Results and discussion

Of the 119 strains, 86 were phenotypically identified to the species level and 33 of them as *Aeromonas* sp. The GCAT gene, described as a specific probe for the genus *Aeromonas* [10] was amplified and revealed that only 75.6% (90/119) of the strains belong to the genus *Aeromonas*. Ten randomly selected strains, negative for the GCAT gene, were analysed by 16S rDNA-RFLP and were further confirmed as not belonging to the genus *Aeromonas* (data not shown). Sequencing of the 16S rRNA gene was performed on 5 of those strains and 4 were identified as belonging to the *Vibrio splendidus* group and 1 as *Plesiomonas* sp. (GenBank accession nos.: FN645426–FN645430). Misidentification of *Vibrio* spp. as *Aeromonas* has been described in other studies [10,33,38]. With the 16S rDNA-RFLP identification method 73/90 (76.6%) strains were identified as belonging to known species, 16 showed the mixed pattern of *A. bestiarum*/*A. salmonicida*/*A. piscicola* and 1 strain showed the common pattern for *A. caviae*/*A. aquariorum* already observed in other studies [6,13,28] (Table 1 and S1). The identity of these 17 strains and of 36 additional strains was investigated by sequencing the *rpoD* gene (Table 1 and S1, Fig. S1). The phenotypic and genetic results of only the 90 confirmed *Aeromonas* strains is shown in Table 1 and S1 and Fig. S1. The strain with the *A. caviae*/*A. aquariorum* RFLP pattern was confirmed as belonging to *A. caviae* (Table 1 and S1, Fig. S1). Interestingly, 1 strain was identified as *A. tecta* [11] on the basis of the *rpoD* gene, and showed a novel species-specific 16S rDNA-RFLP pattern verified after testing the type strain of *A. tecta* and two reference strains (Figs. S1 and S2). This strain was isolated from a diseased bogue fish (*Chondrostoma comun*) and is the first report of this species in this kind of fish, and only the second report from any fish since its recent description [11]. Of the 16 strains that showed the mixed pattern of *A. bestiarum*/*A. salmonicida*/*A. piscicola,* 9 belonged to *A. bestiarum*, 3 to *A. salmonicida* and 4 to the new species, *A. piscicola,* being these 4 strains included in its recent description [6]. Surprisingly the sequences of the *rpoD* gene enabled us to recognize that among the strains showing the RFLP pattern of *A. salmonicida,* 5 (26.3%) in fact belonged to *A. piscicola* as did 1 of the 6 strains with the pattern of *A. bestiarum* (Table 1 and S1, Fig. S1). These 6 strains represent the first records of *A. piscicola* and the first recovery from bogue fish since its recent description [6]. The mentioned confusion occurred because the 16S rDNA analysis performed in the description of *A. piscicola* showed that some strains of this species had identical 16S rDNA sequences to *A. salmonicida,* while others had identical 16S rDNA sequences to *A. bestiarum* [6]. Furthermore, these 3 species may share *rrn* operons of the 16S rRNA gene and will therefore show a similar 16S rDNA-RFLP pattern. Consequently, they can only be correctly separated by sequencing housekeeping genes.

After genetic identification (16S rDNA-RFLP and *rpoD*) the strains of each species found were: 23 *A. sobria* (25.5%), 17 *A. salmonicida* (18.8%), 15 *A. hydrophila* (16.7%), 14 *A. bestiarum* (15.6%), 10 *A. piscicola* (11.1%), 6 *A. media* (6.7%), 2 *A. eucrenophila* (2.2%), 1 *A. encheleia* (1.1%), 1 *A. tecta* (1.1%) and 1 *A. caviae* (1.1%), highlighting the great diversity of the species involved in fish disease (Table 1 and S1, Fig. S1).

Biochemical vs. genetic identification

Without taking into consideration the misidentification as other genera a correct phenotypic identification occurred in only 32 (35.5%) of the 90 strains (Table 1 and S1). Only 14 (21.8%) of the 64 phenotypically identified *A. hydrophila* strains belonged to this species while the rest belonged to 9 other species (Table 1 and S1). These incongruent results are comparable to those obtained in previous studies using strains from other origins [9,32], further corroborating what we emphasised earlier [12,24,32,38] that the importance attributed to *A. hydrophila* is a consequence of erroneous identification. In contrast to these discrepancies, coincident results were obtained for 88% (15/17) of the *A. salmonicida* strains, the exception being 2 atypical not-pigment-producing mesophilic isolates (R64, R67). Phenotypic identification was proven to be reliable for psychrophilic *A. salmonicida* isolates but not for the mesophilic strains within