Characterisation of *Aeromonas* species isolated from diseased fish using ERIC-RAPD markers

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**Abstract.** *Aeromonas* spp. are Gram negative bacteria which are pathogenic to fish, amphibians and also humans. For this study 18 strains of *Aeromonas* were isolated from healthy or diseased fish and water samples collected from several areas of the Vidharbha region of Maharashtra state, India, for identification and characterization. *Aeromonas* isolates were characterized by Enterobacterial Repetitive Intergenic Consensus (ERIC) PCR and Random Amplified Polymorphic DNA (RAPD). ERIC and RAPD analysis revealed genetic diversity in the *Aeromonas* genus as judged by comparing banding patterns. Clusters were formed by the unweighted pair group method with arithmetic (UPGMA) averages applied, which correlate with the genetic information of the species. Construction of phylogenetic dendograms based on Jaccard's coefficient revealed that isolates can be divided into different clusters and three-dimensional plots extracted by principal coordinate analysis using UPGMA of *Aeromonas* spp.

**Keywords:** *Aeromonas* spp., ERIC, Genetic fingerprinting, RAPD.

**INTRODUCTION**

In India, *Aeromonas* spp. are common contaminants in fish, a variety of raw meat, milk and milk products, and other raw foods (Subhaskumar et al., 2006; Porteen et al., 2007). They are widely distributed in the environment, commonly inhabit an aquatic environment and are also part of the normal intestinal microflora of healthy fish (Trust and Sparrow, 1974).

*Aeromonas* is an opportunistic and zoonotically important bacteria belonging to the family *Aeromonadaceae*. Several species, such as *Aeromonas hydrophila*, *A. bestiarum*, *A. sobria*, *A. veronii*, *A. salmonicida*, *A. jandaei*, and *A. allosaccharophila*, have been known to be associated with several diseases, in both warm and cold blooded animals as a result of their virulence and pathogenicity (Gosling, 1995). Motile *Aeromonas* spp. cause a hemorrhagic septicemia in numerous species of cultured and wild freshwater fish such as carp, rainbow trout, brown trout, salmon, eel, carp, channel catfish, tilapia, and goldfish. In humans they cause opportunistic infections and gastroenteritis, chronic diarrhea, wound infections, respiratory tract infections, peritonitis, urinary tract infections and septicemia (Janda and Abbott, 1996; Altwegg, 1999).

The genus *Aeromonas* comprises of several species of gram-negative, rod shaped, motile and non-motile, oxidase and catalase positive, nitrate to nitrite reducing, glucose-fermenting bacteria (Cantas et al., 2012). Owing to the varied nature of these bacteria and their widespread habitat, they have been difficult to classify, making the taxonomy of the genus controversial (Carnahan and Altwegg, 1995). There is therefore a need for a system of identification and characterization of *Aeromonas* isolates to their ecological and clinical importance. Characterization of this genus using ERIC-PCR and RAPD fingerprinting is rapid and sensitive, thereby making the detection and identification of pathogens relatively easy. Structural features within specific community profiles of 18 *Aeromonas* strains isolated from diseased or healthy fish and from water samples are here compared with these molecular techniques. The present investigation therefore represents a possible approach for the characterization of *Aeromonas* spp. at the genomic level and an effort to classify the bacteria at the species level to facilitate the study of contamination by, and pathogenesis of, *Aeromonas* spp.

**MATERIAL AND METHODS**

**Bacterial isolates and Positive cultures** All presumptive strains of *Aeromonas* spp. were isolated from fish (Figure 1) and water samples originating from different areas of the Vidharbha region (Table 1). The putative isolates were then

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Cantas et al., 2012.
subjected to various characterizations which eventually led to their identification. The bacterial strains were maintained on nutrient agar while the growth of *Aeromonas* spp. was confirmed on Brain heart infusion agar and *Aeromonas* selective agar (with ampicillin supplement). The isolates were then subjected to biochemical characterization using the Enterobacteriaceae Biochemical Identification Kit and conventional biochemical tests (Abbott, 2003). *Aeromonas hydrophila* MTCC-1739 and *A. hydrophila* MTCC-646 were used as positive reference strains (Table 1).

**Antibiotic Sensitivity Test** Isolates were screened for sensitivity to a total of 8 antibiotics by a disk diffusion method. The antibiotic sensitivity test was performed with Octadiscs (Himedia, Mumbai) using Mueller-Hinton medium. Mueller-Hinton agar plates were inoculated with 4-hour broth culture of isolates. The plates were incubated at 37°C for 24 hours. Results were obtained in the form of zone of clearance (antibiotic sensitivity), measured as diameter of the zone (in millimeters). The following antibiotic discs were used: Ampicillin (A, 10mcg), Co-Trimoxazole, (Co, 25mcg), Tetracycline (T, 25mcg), Penicillin (P, 10units), Norfloxacin (Nx, 10mcg), Nalidixic acid (Na, 30mcg), Cefuroxime (Cu, 30mcg), and Pipemidic acid (Pa, 20mcg).

**Genomic DNA Isolation** Colonies of *Aeromonas* spp. were grown in Luria-Bartani Broth and incubated at 30°C with continuous shaking at 100 rpm for 24 hours. A 1.5ml aliquot of culture was taken from the incubated bacterial medium and then centrifuged at 13000g for 2 minutes. The supernatant was discarded and the sediment (pellet) was used for DNA extraction. The protocol was followed as per the Bacterial Genomic DNA Prep Kit (Bangalore Genei). The extracted DNA was used for RAPD and ERIC-PCR analyses.

**Enterobacterial Repetitive Intergenic Consensus (ERIC) PCR** The ERIC PCR method utilizes primers complimentary to ERIC sequences in the genomic DNA of *Aeromonas* spp. Two specific primers were used correlating to ERIC 1 (R) and ERIC 2 sequences (Table 2). PCR amplification were performed in a 25 μl reaction volume containing 17.5 μl sterile distilled water, 2.5 μl 10x PCR buffer without MgCl₂, 3.75 mM of MgCl₂, a 200μM deoxyribonucleotide phosphate (dNTP) mix, 50 pmol of each primer, 2U Taq DNA polymerase and 1μl template DNA. The cycling conditions were an initial denaturation at 95°C for 7 minutes, followed by 30 cycles of denaturation at 90°C for 30 seconds, annealing at 52°C for 1 minute, and extension at 65°C for 8 minutes, then a final extension at 68°C for 16 minutes.
Characterisation of Aeromonas species

Figure 2. Antibiotic sensitivity test of isolates of Aeromonas spp. using Octadisc (Himedia). ‘Zone’ of clearance of antibiotic sensitivity against isolates measured in diameter (mm).

Random amplified polymorphic DNA (RAPD) PCR  Ten primers were used for RAPD typing (Table 2). Amplification for RAPD PCR was conducted in a 25μl reaction mixture containing 10x reaction buffer, a 200 μM deoxynucleotide triphosphate (dNTPs mix), 50pmol of primer, 1.5mM of MgCl2, template DNA, and 1.5U of Taq polymerase. The reaction mixture was denatured at 94ºC for 2 minutes, followed by 35 cycles of denaturation at 94ºC for 1 minute, annealing at 36ºC for 1 minute, with an extension at 72ºC for 2 minutes, and a final extension at 72ºC for 10 minutes.

Agarose gel electrophoresis  The amplification products were separated by electrophoresis on a 1.5% agarose gel (w/v) in Tris-Borate buffer (0.089M Tris, 0.089M boric acid, and 0.002M EDTA, pH8), stained with ethidium bromide (1.6 mg/ml) and visualized under a UV light transilluminator. Sizes of the PCR products were determined by comparison with 100bp DNA ladder.

Computer assisted analysis of genomic fingerprints  The genomic fingerprints obtained were compared for similarity by visual observation of the band patterns according to the presence or absence of each band in each isolate. Computer analysis was carried out by NTSYS-pc (Numerical Taxonomy System, version 2.0) software. A dendogram and PCA was constructed by the unweighted pair-group method with average linkages (UPGMA).

RESULTS

The results of the present investigation of Aeromonas isolates, on the basis of biochemical and Gram staining, showed only a few characteristics which exhibited uniformly in all isolates: gram negativity, motility, oxidase positivity and catalase positivity, ability to fermentat D-glucose, nitrate reduction and ampicillin resistance. Further, the selective media plates with yellowish-green or yellow colored colonies were exposed to iodine vapors which produced a clear zone around the colonies of Aeromonas spp. The confirmatory biochemical tests are oxidase positivity, resistance to ampicillin (10 mg/L), fermentation of dextrin and Mannitol, ability to grow in 1% tryptone water containing 0% but not 6% NaCl, resistance to 0129 (2,4-diamino-6,7 Diisopropyl pteridine) phosphate (50mg/L) and hydrolyse arginine. The production of H₂S showing as a black color was also found to be a typical characteristic allowing identification of A. hydrophila. The results shown in Figure 2 represent the antibiotic sensitivity test results of selected strains of presumptive Aeromonas isolates. The results were calculated by taking an average of three replicates, and the zone of clearance, or antibiotic sensitivity, of isolates were measured from the diameter (mm). Our results confirm that Aeromonas spp. are poorly susceptible to Norfloxacin (15mm) and to a lesser extent tetracycline (17mm). Co-Trimoxazole (24mm) susceptibility was similar to that to Nalidixic acid (21mm); Ampicillin and penicillin produced no clear zone, while Cefuroxime (8 mm) and Pipemidic acid (6mm) produced only an insignificant zone of activity.

A total of ten primers (Table 2) were used for RAPD fingerprinting of Aeromonas isolates, though some primers did not result in amplification products for all isolates. The resultant banding patterns (Figure 4) were analysed, and used to produce a dendrogram showing genetic relatedness of 18 strains of Aeromonas, determined using Jaccard’s coefficient and the UPGMA cluster method (Figure 5b). A second dendrogram was produced to show polymorphism and three-dimensional plots extracted by principal Coordinate analysis using UPGMA of Aeromonas isolates (Figure 6).

Distinct ERIC-PCR fingerprint patterns (Figure 3) of

<table>
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<th>Sequence 5' to 3'</th>
<th>References</th>
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<tr>
<td>ERIC-PCR</td>
<td>ERIC 1R</td>
<td>ATGTAAGCTGCTGAGATGC</td>
<td>Versalovic, 1991; Szczuka, 2004</td>
</tr>
<tr>
<td>RAPD</td>
<td>OPA-04</td>
<td>AATTCCGACTCT</td>
<td>Oakey, 1996; Delmare, 2002; Szczuka, 2004; Zulkifli, 2009</td>
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Table 2. Characteristics of molecular methods used for differentiation of Aeromonas spp.
between 3 to 9 amplification bands with similarity coefficient 0.88 were used to produce a dendrogram (Figure 4) indicating variance in 18 isolates of *Aeromonas*. A dendrogram using Jaccard’s coefficient based on the UPGMA cluster method divided into two major clusters, showing genetic relatedness of isolates determined by analysis of ERIC PCR fingerprint patterns (Figure 5a), showed clear polymorphism with similarity ranging from 0.37 to 0.87, indicating genetic variance. Based on cluster analysis and principal coordinate analysis (Figure 6), the results indicated that all accessions could be divided into two major groups and that the clustering pattern was related to their ecological origin.

**DISCUSSION**

The presence and distribution of *Aeromonas* is not well docu-
Aeromonas species

Figure 5. Dendrograms obtained from different isolates of *Aeromonas* spp. with UPGMA based on Jaccard’s coefficient. (a) based on ERIC PCR fingerprint data. (b) based on RAPD data.

Aeromonas is known to be susceptible to all antibiotics active against nonfastidious Gram negative bacteria except β-lactams and resistant to ampicillin, ceohalothin, cefoxitin, and penicillin (Goni-Urriza *et al*., 2000). In this research work a screening test against various antibiotics was done; further research to test for Minimal Inhibitory Concentrations (MIC) are needed in future to assess the clinical relevance of above mentioned strains. On the basis of biochemical and morphological tests, several of these isolates have been demonstrated to be identical, but genetic studies like PCR-based molecular typing have supported the existence of genetic variability among the isolates. By using molecular typing with the aid of RAPD and ERIC-PCR methods a high diversity of polymorphism has been demonstrated between isolates (Szczuka, 2004).

In this study, polymorphism found among the isolates demonstrated that *Aeromonas* species are highly heterogeneous. Genome fragments identified with these novel strategies may be used as genome-specific markers for dynamic
monitoring and sequence-guided isolation of functionally important *Aeromonas* populations in complex communities such as fish microflora. As several authors have suggested (Versalovic, 1991; Davin, 1998; Solat et al., 2003; Szczuka, 2004; Subhaskumar, 2006) molecular typing has great discriminating power and could be used in epidemiological studies of *Aeromonas* spp. RAPD analysis of *Aeromonas* is efficient for discriminating between isolates but is not useful for the characterization of strains at the species level (Delmare et al., 2002). The findings from both techniques were shown to be suitable for the differentiation of unrelated strains and useful for epidemiological investigation and population genetic analysis of *Aeromonas* spp. (Szczuka, 2004). Three dimensional (3D) principal component analyses were constructed to provide another means of testing the relationships between isolates, these are known to be less sensitive to distances between close neighbors but to represent more accurately between clusters associations among subgenera, as also revealed by 3D principal component analyses (Sneath and Sokal, 1973).

In conclusion, genotyping showed considerable differentiation along the subgeneric boundaries and within the genus *Aeromonas*, and by using these methods we can contribute tremendously to the understanding of overall distribution patterns of genetic variation. As a result of this study, we know that fish and water samples taken from reservoirs in the Vidharbha region were considerably contaminated with *Aeromonas*, so clearly fish were at risk of related diseases which also pose a threat to human health. Prevention of disease is always preferable and more cost effective than treatment of disease outbreaks. Preventive medicine programs should be designed to minimize stress, maintain the best water quality possible, and minimize exposure to infectious agents. Further research is needed to identify and study the responsible determinants of virulence.

Figure 6. Three-dimensional plots extracted by principal Coordinate analysis using UPGMA of *Aeromonas* spp. isolates. (a) 3D plot using ERIC markers. (b) 3D plot using RAPD markers.
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