

Identification and Typing of *Aeromonas Hydrophila* through 16S rDNA-PCR Fingerprinting

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Abstract

16S rRNA is a conserved biomolecule within a cell. Sequencing data was analyzed and examined by sequencing the corresponding 16s rDNA regions of isolates from different sources. Specific culture media and different biochemical tests primarily confirmed isolates as *Aeromonas hydrophila*; those were designed and tested following PCR assays and identified by 16S rDNA gene sequence analysis. To automate the method few online and offline computational tools have been used. The method has proven useful for identification of *Aeromonas* species. Our results emphasize the need to take into account the intragenomic diversity of the 16S rRNA gene. Different software and programme used in this study are freely available online in different website.

Keywords: *Aeromonas Hydrophila*; 16S rDNA; Gene sequencing; Molecular typing; Phylogenetic analysis

Introduction

Aeromonas species are widely distributed throughout the world and causative pathogen for variety of fish, animal and human diseases. Particularly relevant to aquaculture is the species (Martínez-Murcia et al. [1]). *Aeromonas hydrophila* are gram-negative, oxidase-positive, facultative anaerobic, glucose fermenting rods belongs in the family *Aeromonadaceae*.

Raghava et al. stated that the identification and classification of microorganisms are of fundamental importance in microbiology [2]. Classical methods are basically dependent on phenotypic data. These are also time consuming and expensive. Various fingerprinting methods can be broadly classified DNA fingerprinting by restriction enzyme analysis of the genomic DNA on pulse field gel electrophoresis, for a particular region of the genome the repetitive DNA sequence can be used as a probe, DNA-DNA hybridization and amplification of particular region of genome by PCR followed by restriction analysis. All of these methods of genomic DNA have been the subject of analysis (Grimont and Grimont [3], Huys et al. [4] and Skuce et al. [5]). *A. hydrophila* may exhibit slightly different characteristics depending on the environment from which they were isolated. However, all of these methods have been used for epidemiological studies or to differentiate between strains of the same species or to confirm the species of the genus.

Ribosomes are a part of the translational machinery of a cell and rRNA plays a vital for cellular growth, function and survival. Consequently, the primary, secondary and tertiary structures of rRNA molecules have been conserved during evolution (Gutell [6]). 16S rRNA analysis have stated the primary structure of rRNAs and comparative oligonucleotide consists of highly conserved regions interspersed by regions of moderate to low homology within related species (Gopo et al. [7]) Direct sequencing of the 16S rRNA gene is generally accepted as a stable and specific marker for bacterial identification (Marchandin et al. and Woese [8,9]). The 16S rRNA gene is often organized as a multigene family, with the copy number ranging from 1 to 15 rRNA (*rrn*) operons in the bacterial genomes (Coenye and Vandamme [10]). Usually the maximum size of the 16S rRNA is 1500 bp. Despite the conserved nature of rRNAs, they vary in size and in the organization of the spacer as well as variable regions within the rRNA. However, the size

of the 16S rRNA, sequence conservation during evolution confirmed its importance. Classification of bacteria through this technique also been proven that this may be effectively useful for phylogenetic identification at various levels. To believe the usefulness of this fingerprinting technique *Aeromonas spp.* was chosen.

Materials and Methods

Bacterial strains and culture conditions

Samples were collected from different area and sources of West Bengal, India. Eighty two of isolates were predominantly collected from water of pond, sea and river; raw meat of chicken and mutton; raw cow milk and Fishes as Rohu (*Labeo rohita*), Katla (*Katla katla*), Hilsha (*Hilsha hilsha*) and Mackerel. Out of the 82 strains, 39 strains form fishes were identified phenotypically and biochemically. Strains were also identified by the 16S rDNA-PCR technique included in the study. All isolates were grown on a specific media for *A. hydrophila* as Rimler Shotts agar (Himedia) at 37°C for 38 hours and followed by inoculation of single colony in the Soybean casein digestive media (TSB- Difco) for further growth and studied for genomic characterization, sequencing and typing.

Biochemical studies

Biochemical tests were done in all strains by “Automated Microbial Analyzer” (Biolog, US). The metabolic profiles were compared automatically using the Biolog microtiter plates with the MicroLog GN database. Biolog identifications were reported if the similarity index of the genus or species was 0.5 or greater after 24 hr of incubation. Test samples were incubated under the same conditions as used. All tests

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were carried out in duplicate, and appropriate positive and negative controls were included.

Bacterial DNA extraction

Bacterial biomass from 10 ml of culture was collected by centrifugation for 5mins at 10000x g and resuspension in 400 µl of TE buffer (10 mM Tris- HCl, 1 mM EDTA, pH 8.0). Then the cells were lysed by the addition of 200 µl of 10 mg ml⁻¹ lysozyme and incubation at 37°C for 60 min. Then the preparation was incubated for 10 min with 40 µl of 10 mg ml⁻¹ proteinase K at room temperature followed by the addition of SDS to a final concentration of 1% and incubation at room temperature until the preparation was clear. Eighty µl of 0.5 M EDTA was added and mixed gently and the solution was deproteinated by sequential phenol: chloroform: isoamyl alcohol (24:23:1 v/v) extraction. 0.6 volume of isopropanol was added to precipitated DNA and after centrifugation, washed with 70% alcohol and dried it by Maxi Vacuum dryer. Then 500 µl TE buffer (pH 8.0) was added to for storage and for further use. The DNA concentration was estimated by visual comparison with the standard DNA size markers after electrophoresis through 0.8% agarose TAE (tris-acetate EDTA) gels stained with 0.5 mg ml⁻¹ ethidium bromide (Sigma Chemicals Co.).

PCR amplification

The amplification was performed on 1.5 µl DNA extract in 20 µl using High Fidelity PCR System (GeneAmp PCR System 2400). PCR products were visualized by ethidium bromide staining on 1.5% agarose gel electrophoresis. PCR reactions has been carried out by following the series of standardizing experimental protocol as annealing temperature, concentration of MgCl₂, template DNA, Taq DNA polymerase, dNTP's and primers. The PCR reaction components consists of 200 mM dNTP, 20 pico moles of primer, 2 units of Taq DNA polymerase enzyme, assay buffer with working concentration of 1.5 mM MgCl₂, 20-30 ng template DNA in an assay volume of 25 mL. These concentrations were determined by a series of preliminary standardizing experiments.

16S rDNA PCR amplification

Based on morphological and biochemical characteristics a number of colonies representing all recovered aeromonads in this study were chosen for identification based on 16S rDNA analysis. DNA extraction was first evaluated by the amplification of the 16S rRNA gene using the gene sequence universal primers: forward primer 27F 5'-AGA GTT TGA TCC TGG CTC AG-3' and reverse primer 1492R 5'- GGT TAC CTT GTT ACG ACT T-3' (Chromous Biotech Ltd., Bengaluru, India) (Stackebrandt et al. [11]). The PCR products were purified by the Exosap treatment in the Molecular Biology Laboratory, Department of Biotechnology, The University of Burdwan, West Bengal. PCR products were sequenced directly from the Xcelris Lab Ltd, Ahmedabad, India.

Results

Biochemical assay

A. hydrophila appeared as deep cream or light yellow colonies with entire margin in the Rimler shotts agar medium. In primary characterization tests, they were gram negative, rod shaped, motile, oxidase positive, fermentative and novobiocin resistant, suggesting that colonies are aeromonads. All isolates were confirmed to the species level as *A. hydrophila* by differential biochemical tests (Table 1).

Analysis of 16S rDNA PCR

Thermal cycling was performed with GeneAmp PCR System 2400.

Test	Reaction of isolates					
	AH1	AH2	AH3	AH4	AH5	AH6
Gram stain						
Motility	-	-	-	-	-	-
Oxidase	+	+	+	+	+	+
Catalase	+	+	+	+	+	+
O/F test	+	+	+	+	+	+
Fermentation	+	+	+	+	+	+
Sugar Glucose	+/+	+/+	+/+	+/+	+/+	+/+
Sucrose						
Lactose	+	+	+	+	+	+
Mannitol	-	-	-	-	-	-
Maltose	+	+	+	+	+	+
Citrate	+	+	+	+	+	+
utilization	+	+	+	+	+	+
Starch	+	+	+	+	+	+
hydrolysis	+	+	+	+	+	+
Gelatin	+	+	+	+	+	+
hydrolysis	+	+	+	+	+	+
Nitrate reduction						

Table 1: Physiological and Biochemical Characteristics of isolates in the study.

Sl. No.	Sample ID	BLAST Details				
		Organism	GenBank Accession No.	Max Score	Query Coverage	Max Identity
01.	AH1	<i>A. hydrophila</i> strain PIA31 16S ribosomal RNA gene	JN559379	1688	100%	96%

Table 2: 16S rRNA gene sequence accession no. from NCBI GenBank.

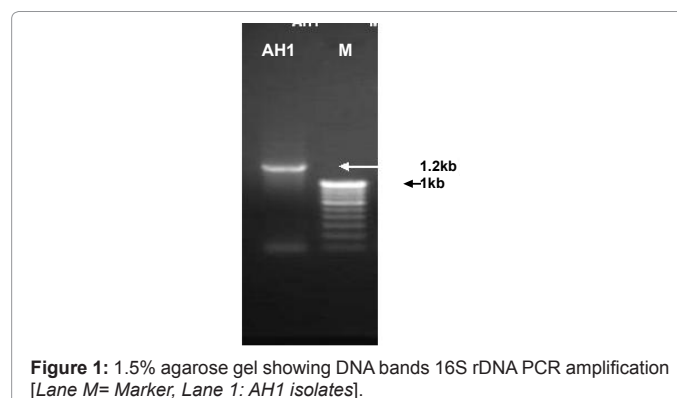
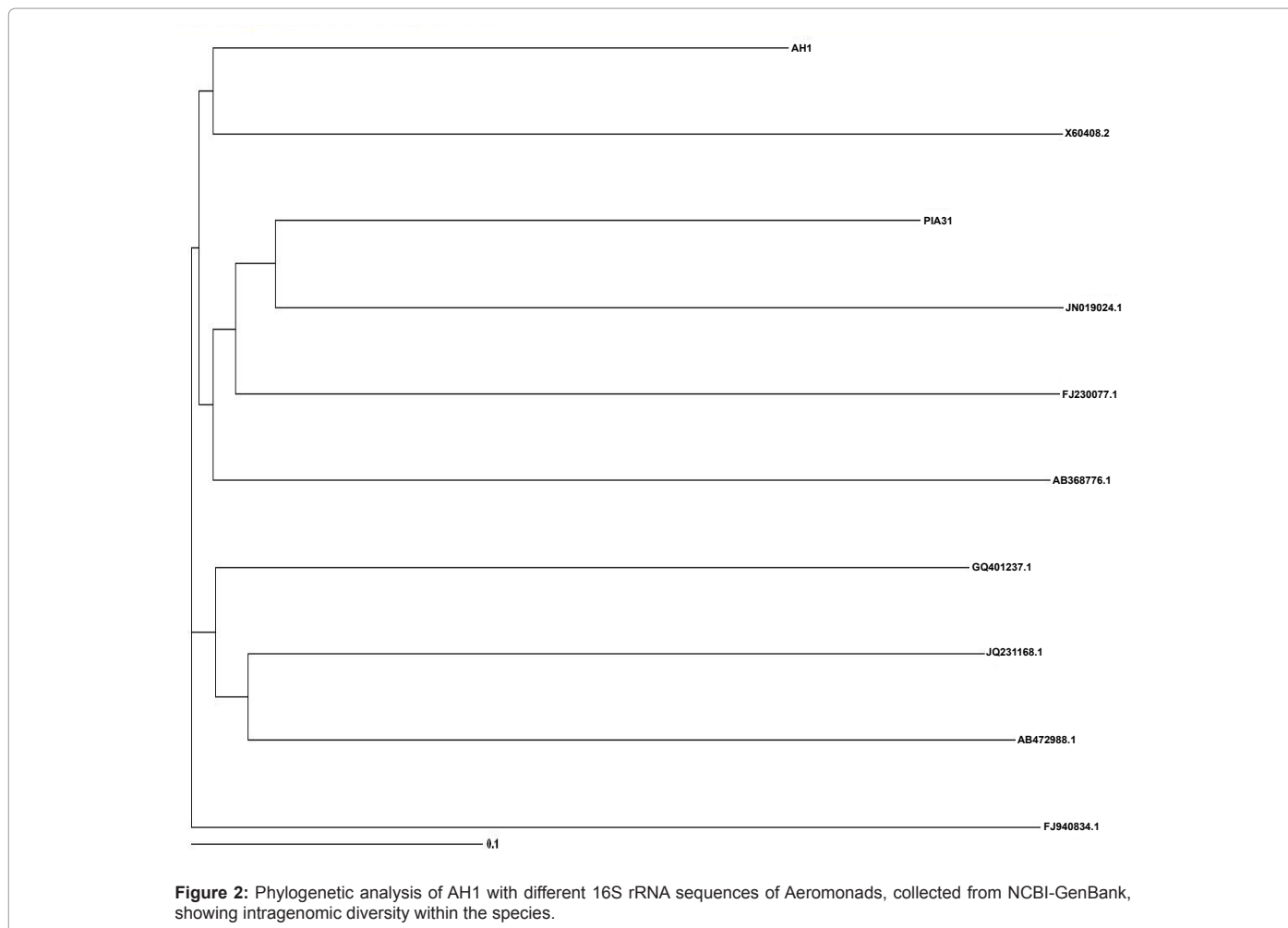


Figure 1: 1.5% agarose gel showing DNA bands 16S rDNA PCR amplification [Lane M= Marker, Lane 1: AH1 isolates].

Amplification reactions were performed in a 25 µL volume, containing: 20 mmol/L Tris-HCl (pH=8.4), 50 mmol/L KCl, 2.0 mmol/L MgCl₂, 200 µmol/L of dNTPs, 1 µmol/L of each primer, 30 ng of genomic DNA and 1.5 U of Taq DNA polymerase. The temperature profile was as follows: initial denaturation at 95°Cx3 min, 35 cycles of denaturation at 94°Cx1 min, annealing at 55°Cx1 min, and extension at 72°Cx2 min, and final extension at 72°Cx3 min. The PCR products were analyzed by electrophoresis on 1.5% agarose gels and stained with ethidium bromide. 100 bp ladder was used for evaluating the size of amplicons. The DNA bands were visualized and documented using a Gel documentation system (Biorad Gel Doc. 2000 system).

Sequencing and computer analysis 16S rDNA genes

For the validation of *Aeromonas* identification, chosen strain was identified by 16S rDNA PCR pattern analysis on 1.5% agarose gel electrophoresis and those were selected for sequencing (Figure 1). In total, the PCR amplified products were sequenced (Figure 2) from Xcelris Labs, Ahmedabad, India. Species identification and similarity



calculations were performed, comparing sequences of approximately 1500 bases with sequences available in GenBank using BLAST network services (Altschul et al. 1997 [12]). Phylogenetic relationship was outlined by online PHYLIP 3.69 version and found similarity index, distance between all isolates and maximum likelihood pattern analysis. Strain identified as *A. hydrophila* with percentile match, based on NCBI-BLAST and sequences were compared. The 16S rDNA sequences in this study were submitted to the GenBank database by using Sequin software under nucleotide accession number of AH1 as JN559379 (Table 2).

Phylogenetic analysis of *Aeromonas spp.* based on 16S rDNA sequence

Consensus sequences were aligned using ClustalW for Multiple Sequence Alignment (Thompson et al. [13]), available from GenomeNet Computation service from Kyoto University Bioinformatics Center (<http://align.genome.jp>). A PHYLIP generated DNA distance matrix (dnadist) after little modification (Hall [14]). The tree shows the genetic heterogeneity and distance within the species, because isolates were collected from different sources and genetic variation either deletion or addition of nucleotides has been appeared, however all six isolates were *A. hydrophila*, isolated from different sources.

Discussion

The taxonomy of *Aeromonas* remains difficult because several

approaches have been attempted for classification of the phylogenetic relationships among the species of the genus (Huys et al [4] and Martínez- Murcia [15]), An interesting aspect observed in the present study was that *A. hydrophila* showed mesophilic (Austin B et al., 1998, Austin DA et al., 1989, Marchandin et al. [8], Martínez-Murcia [15], Popoff [16]). Classical tests used to separate the subspecies of *A. hydrophila* from each other are motility and indole production (Popoff [16], Pavan et al. [18] and Wiklund and Dalsgaard [19]). However, these properties do not proved as useful for that purpose in our study. Motility only yielded the expected results for the type and reference strains. Evolutionary conservation and the uniqueness of the ribosomal RNA allow a broad spectrum of applications. Grimont and Grimont [3] described the importance of the 16S rRNA-based probes as taxonomic tools. This method has shown discriminatory properties in the identification up to the species level and in the typing of other bacteria. Furthermore, automated DNA sequencing is beyond the means of many laboratories to sequence large numbers of microorganisms. However, the need to protect biodiversity from the negative impact of globalization has revived the demand for simple yet comprehensive and reliable methods of identification. In the present study, we approached the use of the properties of the 16S rDNA for phylogenetic identification from a different perspective. The objective of the present study was to provide a quick identification tool but not to carry out a taxonomic study.

Views were recognition of the heterogeneity detected in the

sequences is important to avoid strain misidentification and faithful to the acknowledgement of diversity. Clarridge [20] drawn “sequesvars” as different ways to record sequence variations in relation to the type strain. This approach can also be adapted for recording microheterogeneities in sequences deposited in databases as designating the position and base change and combining this information with the percentage of positions showing microheterogeneities.

Conclusion

To draw a keen biosecurity protocol molecular typing, molecular diagnosis and phylogenetic relationships within the species can be able to overcome threats and control the disease outbreaks of *Aeromonas* species. These data and methodology has been proving as a great and advance molecular tool. An increasing number of reports on *Aeromonas spp.* associated with health problems; drinking water may represent an important source of pathogenic *Aeromonas spp.* (Handfield et al. [21]). The isolation of *Aeromonas spp.* from samples collected from the distribution network in this study could be attributed to the ineffective treatment strategy employed in the system, which may enhance the survival of the organism as suspended or as part of the biofilm community. This preliminary study further emphasizes the need to survey the incidence of *Aeromonas spp.* in different sources in relation to *Aeromonas*-associated pathogenicity in West Bengal, India. Moreover, studies on *Aeromonas* should include assessment of the virulence factors of the predominant strains. Our results emphasize the need to take into account the intragenomic diversity of the 16S rRNA gene.

In conclusion, these data apparently cover the majority of *Aeromonas* species that can be discriminated by 16s rDNA targeted primers. Our study is aimed towards the quantification of members of the genus *Aeromonas* as indicators in environment. *A. hydrophila* was identified as the most prevalent species in the waterways, food sample like raw milk and raw meat in West Bengal, India.

Authors' contribution

The research work is really handled and designed by Agniswar Sarkar. Part of computational work was handled by Avijit Patra. Mousumi Saha helped to conclude and finish the work and Pranab Roy contributes his intellectual input for the study.

References

- Martínez-Murcia AJ, Soler L, Saavedra MJ, Chacón MR, Guarro J, et al. (2005) Phenotypic, genotypic, and phylogenetic discrepancies to differentiate *Aeromonas salmonicida* from *Aeromonas bestiarum*. *Int Microbiol* 8: 259-269.
- Raghava GP, Solanki RJ, Soni V, Agrawal P (2000) Fingerprinting Method for Phylogenetic Classification and Identification of Microorganisms Based on Variation in 16S rRNA Gene Sequences. *Biotechniques* 29: 108- 116.
- Grimont F, Grimont PA (1986) Ribosomal ribonucleic acid gene restriction patterns as potential taxonomic tools. *Ann Inst Pasteur Microbiol* 137: 165-175.
- Huys G, Coopman R, Janssen P, Kersters K (1996) High-resolution genotypic analysis of the genus *Aeromonas* by AFLP fingerprinting. *Int J Syst Bacteriol* 46: 572-580.
- Skuce RA, Brittain D, Hughes MS, Beck LA, Neil SD (1994) Genomic fingerprinting of *Mycobacterium bovis* from cattle by restriction fragment length polymorphism analysis. *J Clin Microbiol* 32: 2387-2392.
- Gutell RR (1992) Evolutionary characteristics of 16S rRNA and 23S rRNA structures, 243-309. In H. Hartman and K. Matsuno (Eds.), *The Origin and Evolution of the Cell*. *World Scientific Publishing*, Singapore.
- Gopo JM, Melis R, Filipka E, Meneveri R, Filipski J (1988) Development of a *Salmonella*-specific biotinylated DNA probe for rapid routine identification of *Salmonella*. *Mol Cell Probes* 2: 271-279.
- Marchandin H, Teyssier C, Siméon De Buochberg M, Jean-Pierre H, Carriere C, et al. (2003) Intra-chromosomal heterogeneity between the four 16S rRNA gene copies in the genus *Veillonella*: implications for phylogeny and taxonomy. *Microbiology* 149: 1493-1501.
- Woese CR (1987) Bacterial evolution. *Microbiol Rev* 51: 221-271.
- Coenye T, Vandamme P (2003) Intragenomic heterogeneity between multiple 16S ribosomal RNA operons in sequenced bacterial genomes. *FEMS Microbiol Lett* 228: 45-49.
- Stackebrandt E, Murray RGE, Truper HG (1988) *Proteobacteria* classis nov., a name for the phylogenetic taxon includes the 'purple bacteria' and their relatives'. *Int J Syst Bacteriol* 38: 321-325.
- Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, et al. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 25: 3389-3402.
- Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The Clustal_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* 25: 4876-4882.
- Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* 41: 95-98.
- Martínez-Murcia AJ (1999) Phylogenetic positions of *Aeromonas encheleia*, *Aeromonas popoffii*, *Aeromonas* DNA hybridization group11 and *Aeromonas* group 501. *Int J Syst Bacteriol* 49: 1403-1408.
- Popoff M (1984) Genus III. *Aeromonas* Kluyver and Van Neil 1936, 398AL. In: Krieg NR, Holt JG (ed) *Bergey's manual of systematic bacteriology*. Baltimore, Williams & Wilkins, 545-548.
- Martínez-Murcia AJ, Antón AI, Rodríguez-Valera F (1999) Patterns of sequence variation in two regions of the 16S rRNA multigene family of *Escherichia coli*. *Int J Syst Bacteriol* 49: 601-610.
- Pavan ME, Abbott SL, Zorzópulos J, Janda JM (2000) *Aeromonas salmonicida* subsp. *pectinolytica* subsp. nov., a new pectinase-positive subspecies isolated from a heavily polluted river. *Int J Syst Evol Microbiol* 50: 1119-1124.
- Wiklund T, Dalsgaard I (1998) Occurrence and significance of atypical *Aeromonas salmonicida* in non-salmonid and salmonid fish species: a review. *Dis Aquat Organ* 32: 49-69.
- Clarridge JE (2004) Impact of 16S rRNA Gene Sequence Analysis for Identification of Bacteria on Clinical Microbiology and Infectious Diseases. *Clinical Microbiology Reviews* 17: 840-862.
- Handfield M, Simard P, Letarte R (1996) Differential media for quantitative recovery of waterborne *Aeromonas hydrophila*. *Appl Environ Microbiol* 62: 3544-3547.