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# Application of Repetitive Extragenic Palindromic sequence (REP) as molecular marker for biotyping *Aeromonas* strains isolated from surface and sub- surface soil sediments

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**ABSTRACT**

*Aeromonas* species are ubiquitous bacteria in terrestrial and aquatic milieus. They are becoming renowned as enteric pathogens of serious public health concern as they acquire a number of virulence determinants that are linked with human diseases, such as gastroenteritis, septicaemia, and skin diseases. Identification of *Aeromonads* to the species level is problematic and complex due to their phenotypic and genotypic heterogeneity. With the aim of clarifying taxonomic relationships among the strains, Repetitive Extragenic Palindromic sequence (REP) was used as molecular marker for biotyping *Aeromonas* strains isolated from surface and sub- surface soil sediments from Forest ecosystem. Out of a collection of 120 strains isolated from surface and sub-surface soil sediments, 15 strains were confirmed as *Aeromonas* based on phenotypic fingerprinting obtained from 69 biochemical tests. Genetic diversity among *Aeromonas sp*. was determined by analysing their REP fingerprint patterns using NTSYS pc software package and UPGMA cluster method. Considerable amount of genetic diversity was observed among the isolated strains. Furthermore, dendrogram demonstrated the existence of distinctive clusters at various similarity levels. These fingerprinting techniques can therefore be used to recognize the relatedness of strains derived from clinical and environmental isolates, which helps in further characterization of factors/genes responsible for the disease.



## Introduction

The *Aeromonads* are Gram-negative, rod-shaped, facultative anaerobic, nonspore forming bacteria that are autochthonous and widely distributed in terrestrial and aquatic environments [Daskalov et al, 2006].The genus is made up of psychrophiles and mesophiles and causes different kinds of diseases to many warm and cold-blooded animals. *Aeromonas* species are found globally in surface water, ground water, chlorinated drinking water, nonchlorinated drinking water, bottled mineral water [Villari et al, 2003;Chareut et al, 2001;El-Taweel et al, 2001] and broad range of foods [MacMohan et al, 2001]. They are found in the intestinal tract of humans and animals, raw sewage, sewage effluents, sewage contaminated waters, and activated sludge [Go ni-Urriza et al, 2000;Khajanchi et al, 2010].Several analyses on the taxonomy and nomenclature of *Aeromonas* genus have been carried out over the years [Colwell et al, 1986; Abbott et al, 1998;Abbott et al, 2003]. *Aeromonas* species are known as causative agents of a wide spectrum of diseases in man and animals [Ghenghesh et al, 2008]. Some studies have shown that some motile *Aeromonas* species are becoming food and waterborne pathogens of increasing importance [Ara' ujo et al, 2002; Ansari et al, 2011]. They have been associated with several food-borne outbreaks and are progressively being isolated from patients with traveller's diarrhea [Graevenitz et al, 2007]. Presently, as a putatively emerging enteric pathogen, *Aeromonas* species have the inherent capability to grow in water distribution systems, especially in biofilms, where they may be resistant to chlorination [Chauret et al, 2001]. Also, *A. hydrophila* is listed in the Contaminant Candidate List, and Environmental Protection Agency Method 1605 has validated its detection and enumeration in drinking water system [USEPA]. Clinical and environmental *Aeromonas* sp. isolates secrete many extracellular products, such as hemolysins, enterotoxins, and proteases. Studies conducted by Kühn et al. (Kuhn et al, 1997) showed that some isolates of a given species produce virulence factors more frequently than others. These findings indicate that the virulence within the genus *Aeromonas* might be a clonal property and only some clones may be responsible for progressive disease. However, there have been no studies that would have determined clonal structure within *Aeromonas* spp. and the spread of specific clones in human population and in the environment.[Szczuka et al, 2004].

Dispersed repetitive DNA sequences have been described recently in eubacteria. PCR based DNA fingerprinting methods such as RAPD and rep-PCR [Lows et al, 1998] provide a practical and effective means to examine genotypic diversity among *Aeromonas* species.

Our study was undertaken to recognize the clonal relatedness of strains derived from surface and sub-surface soil sediments collected from Forest soil and to identify and characterize a consortium of *Aeromonas* strains isolated from forest soil and to asses their diversity by REP-PCR approach.

## Materials and Methods

### Sampling site

Soil samples were collected from fifteen sites in forest area of Raisen district, Madhya Pradesh which lies in central part of India. Their geographical origin and sources are located in the forest area of Raisen district (Raisen District, Division

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Bhopal), 47 Km away from Bhopal (Table 1). Raisen district lies in the central part of Madhya Pradesh. The district is situated between the latitude $22^0$47' and $23^0$33 north and the longitutde $77^0$21' and $78^0$49' east. Raisen district is dominated by tropical dry deciduous type of forest with the predominance of teak trees Climate is tropical dry and wet type with maximum temperature of $42^0$C and minimum $5^0$ Celsius. It has average rainfall of 1200mm.Eight samples contained surface soil (SS) and seven samples contained soil at 20 cm depth (DS). Surface litter was scraped away and soil was passed through 2 mm sieve to have homogenous particles. Samples were stored in presterilized HDPE bags at $4^0$C.

**Bacterial strains and their identification**

Fifteen strains of *Aeromonas* spp. used in this study were isolated from 15 sampling sites of Forest soil. For isolation of strains serial dilution of homogenised samples was made in phosphate buffer. Briefly, 10 g soil samples were suspended in PBS and well stirred, 10 ml soil derived supernatant was firstly co cultured with 90 ml LB medium for 24 hours. Bacterial suspension was plated onto selective media agar plates.Colonies of *Aeromonas* spp. grown on Trypticase soy agar at 37°C for 24 h were further confirmed by their phenotypic properties.

**DNA Extraction**

Some colonies were scraped off and suspended in 0.85% NaCl. After centrifugation, the pellet was resuspended in a lysis buffer and incubated after adding protease K. The DNA was extracted using the NucleoSpin C + T kit (Macherey-Nagel, Düren, Germany) according to the procedure established by the manufacturer.

**Estimation of DNA**

**(i) Obtaining the ratio of absorbance at 260nm/280nm: -**

The quality and quantity of the DNA were determined spectrophotometrically at 260 nm. Protein content is deduced at $A_{280}$. A ratio $A_{260}/A_{280}$ up to 1.9 is considered as pure DNA sample (Henery, 1997). DNA recovered was dissolved in 15 ml of saline citrate, 0.5 ml of this solution was added to 4.5 ml of saline citrate and absorbance value was obtained at 260 nm & 280 nm. The DNA is capable of absorbing the UV radiation, and the absorbance ratio i.e., 260nm/280nm (Henery et al, 1997) indicates the presence and concentration of DNA, the value exceeding more than 1.9 indicates that concentration of protein is more in the sample of DNA.

**(ii) The temperature of melting (Tm-value) of isolated DNA:-**

The isolated DNA was dissolved in 15 ml of saline citrate. 0.5 ml of this solution was dissolved in 4.5 ml saline citrate in four sets of test tubes. Each test tube was then treated at $60^0$C, $70^0$C, $80^0$C and $90^0$C for 30 minutes in water bath separately and absorbance was taken at 260 nm (Jain et al, 1998). Tm is defined as the temperature at which steep rise in absorbance is half maximum. Values obtained were then plotted and the Tm-value was calculated.

**(iii) The determination of G+C % of the isolated DNA: -**

The % G+C content was calculated from the following formula:

% G+C= 2.44 (Tm – 69.4) (Marmur & Doty, 1962), Where, Tm = temperature of melting calculated from the graph.

**Characterization of DNA by bio-molecular techniques:**

The REP-PCR method uses primers complementary to REP elements of bacterial genomic DNA (Versalovic et al, 1991). PCR amplification of template genomic DNA results in products of different sizes, presumably reflecting the distance and orientation of endogenous repeats. The following primers were used for REP-PCR: Rep1R-I 5′-(inosine)(inosine)(inosine)(inosine)CG(inosine)CG(inosine)CATC(inosine)GGC-3′, and Rep2-I 5′-(inosine)CG(inosine)CTTATC(inosine)GGCCTAC-3′ (Versalovic et al, 1991). REP-PCR analysis was performed in accordance with previous description (Versalovic et al, 1991). The reaction mixture consisted of 4 µl of 10× reaction buffer [750 mM Tris-HCl (pH 8.8), 200 mM $(NH_4)_2SO_4$, 0.1% Tween 20], a 250 µM concentration of each dNTP, 50 pmol of primer Rep1R-I, 50 pmol of primer Rep2-I, 3.75 mM $MgCl_2$, 100 ng of template DNA, and 2 U of *Taq* polymerase (MBI Fermentas) made up to 25 µl with sterile distilled water. The reaction mixture was denatured for 7 min at 95°C and then subjected to 30 cycles of denaturation for 30 s at 90°C, annealing for 1 min 40°C, extension for 8 min at 65°C, and a final extension for 16 min at 65°C.

**Electrophoresis and data analysis**

The amplification products were electrophoresed in 1.5% agarose gel in Tris-borate buffer (0.089 M Tris, 0.089 M $H_3BO_3$, 0.002 M EDTA). Gene Ruler 100-bp DNA Ladder Plus (MBI Fermentas) was used as a molecular size standard. The gels were stained with ethidium bromide, visualized on a UV light transilluminator, and documented with V.99 Bio-Print system (Vilber Lourmat, Torcy, France). REP-PCR fingerprint patterns were coded in the binary form, and analysed using NTSYSpc package [Rohlf, 1990]. Similarity between fingerprints was calculated with the Jaccord's coefficient. A simple matching coefficient was calculated to construct a similarity matrix and UPGMA algorithm was used to perform hierarchical cluster analysis and to construct a dendrogram.

**Results:**

**Identification of *Arthrobacter* strains**

With the aid of selective media fifteen *Aeromonas* strains (B1 to B15) were isolated from surface and deep soil sediments of forest area of Raisen district. Strains were subjected to various biochemical and physiological tests for further confirmation (Table.2). %G+C content of the isolated Aeromonas strans ranged from. 55.3 % to 64.6 %.

**REP-PCR fingerprinting**

REP- PCR fingerprinting analysis of 15 isolated strains of *Aeromonas* yielded six to nine bands depending upon the size of DNA fragment ranging from 100bp to 1000bp (Fig.1). REP - PCR fingerprinting patterns resulted in phylogenetic clusters using primers 1R (5'-IIIICgICgICATCIggC-3') and forward primer REP 2I (5'-ICgICTTATCIggCCTAC-3') (Versalovic, et. al., 1991) followed by electrophoresis with 1.6% agarose. The cluster analysis of fifteen isolated genomic fingerprinting of *Aeromonas* sp. falls between similarity levels of 59 % (Fig.2). Group B4, B5, B9 shows same position in cluster analysis at 90 % similarity. Another similar group consisting of strains B2 and B3 have a 87 % similarity level. Group consisting of strains B6 & B7 have a similarity level of 75%. The findings of REP fingerprinting matches the observation on the basis of NYSYS pc package UPGMA algorithmic programme preferred for phylogenetic cluster analysis.

**Discussion**

Over the last few years several authors have used molecular typing methods for determination of the clonal structure of several species, e.g., *Neisseria meningitidis* (Caugant et al, 1998), *Corynebacterium diphtheriae* (Gubler et al, 1998; Sulakvelidze et al,1999), *Streptococcus pneumoniae* (Robinson et al, 1998, Hsueh et al, 1999), *Salmonella enterica* (Hilton et al, 1998), *S. enterica* serovar Enteritidis (Lin et al,1996), *Clostridium difficile* (Martirosian **et al, 1995**). *Aeromonas sp.* is widely spread in the environment, especially in surface water and sewage; they also occur in untreated and treated drinking water (Brandi et al, 1996; Janda et al, 1998)

**Table 1. Sampling sites**

| S. No. | Sample No. | Location |
|---|---|---|
| 01. | SS1 | M.P. Vaniki Pariyojna, Area 30 Hect., Raisen Van Mandal, Gramin Sansthan Vikas Programme, Gram Van Samiti, Ratanpur, Year 98 – 99. |
| 02. | SS2 | Gram Betna, NH-86, Shore of Betwa River |
| 03. | SS3 | Gram Sabha "Vanchod" |
| 04. | SS4 | 3 Km from Chiklod towards Bhopal |
| 05. | SS5 | Gram Tilendi, Block Obaidullahganj |
| 06. | SS6 | Bhojpur |
| 07. | SS7 | Gram "Vanchod", Vaikalpic Vrakcharopand Karya 2001 – 2003, Van mandal Obaidullahganj, Parichhetra Chiklod Beet-Varokhar, van Suraksha Samiti, Amoda, Kaksha Kramank: 544, Area 50 Hect. |
| 08. | SS8 | Gram Alli, Chiklod Road |
| 09. | DS1 | Gram Betna, NH-86, Shore of Betwa River |
| 10. | DS2 | Gram Alli, Chiklod Road |
| 11. | DS3 | Sombawa se "Vanchod", Gram Sabha "Vanchod" |
| 12. | DS4 | M.P. Vaniki Pariyojna, Area 30 Hect., Raisen Van Mandal, Gramin Sansthan Vikas Programme, Gram Van Samiti, Ratanpur, Year 98 – 99. |
| 13. | DS5 | Gram "Vanchod", Vaikalpic Vrakcharopand Karya 2001 – 2003, Van mandal Obaidullahganj, Parichhetra Chiklod Beet-Varokhar, van Suraksha Samiti, Amoda, Kaksha Kramank: 544, Area 50 Hect. |
| 14. | DS6 | Gram Tilendi, Block Obaidullahganj |
| 15. | DS7 | 3 Km from Chiklod towards Bhopal |

Previously, Moyer et al,1994 reported that two *A. hydrophila* and four *A. caviae* strains isolated from stool specimens collected from people living in the same city exhibited genetic variability. Hänninen et al, 1995 indicated that strains within *A. veronii* biotype sobria, *A. caviae*, and *A. hydrophila* had a unique ribotype. In humans, *Aeromonas sp*. are responsible for gastroenteritis, chronic diarrhea, wound infections, respiratory tract infections, peritonitis, urinary tract infections and septicemia (Janda, et. al., 1996). Among *Aeromonas* associated infections of humans *A. hydrophila*, *A. caviae*, and *A. veronii* are the predominating species. Clinical and environmental Aeromonas sp. isolates secrete many extracellular products, such as hemolysins, enterotoxins, and proteases. Occurance of *Aeromonas sp*. in forest soil might be from run off water from nearby inhabited areas.

This study emphasizes the importance of using more than one method for the correct identification of *Aeromonas* strains**.** Above studies indicate that REP-PCR analysis yields relatively small number of bands and that too are not distinguishable. This suggests that the repetitive extragenic palindromic sequences are not widely distributed in *Aeromonas* sp. genome. For characterization of *Aeromonas* strains REP-PCR should be coupled to other charaterization techniques like ERIC-PCR, BOX-PCR etc. Secondly it is suggested that comparison should be made between clinical and environmental isolates which may lead to detection of factors responsible of disease outbreak. Furthermore, These fingerprinting techniques can therefore be used to recognize the relatedness of strains derived from clinical and environmental isolates, which helps in characterization of strains responsible for the disease.

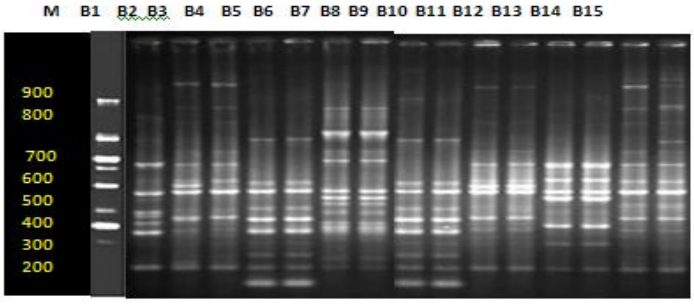


**Fig 1. REP PCR fingerprint of isolated strains of *Aeromonas sp.* isolated from forest soil of Raisen district.**

**M – Marker**
**B1 to B8 – Surface soil isolate**
**B9 to B15 – Deep soil isolate**

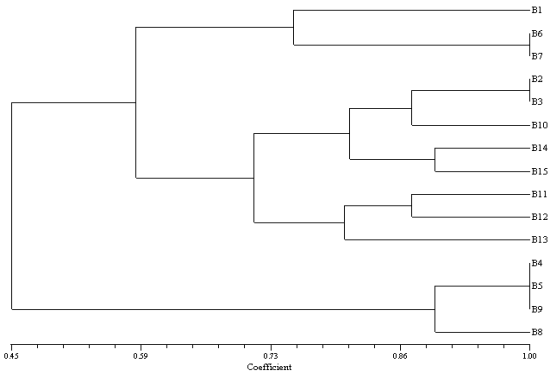


**Fig 2. Dendrogram showing genetic relatedness of 15 strains of *Aermonas* from forest soil of Raisen District determined by analysis of REP-PCR fingerprint patterns using Jaccord's similarity Coefficient and UPGMA cluster method.**

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**Table 2. Biochemical tests as displayed by *Aeromonas* strains**

| Biochemical Test | Result |
|---|---|
| **Colony Morphology** | |
| Configuration | Round |
| Margin | Entire |
| Elevations | Convex |
| Surface | Smooth |
| Density | Translucent |
| Pigments | - |
| Gram's Reaction | -ve |
| Cell shape | Rods |
| Size | Short |
| Arrangement | Single |
| **Characteristics** | |
| Spore(s) | - |
| Capsule | - |
| Motility | + |
| Fluorescence (UV) | - |
| **Growth at Temperatures** | |
| $4^0$C | - |
| $10^0$C | - |
| $15^0$C | + |
| $25^0$C | + |
| $30^0$C | + |
| $37^0$C | + |
| $42^0$C | + |
| $45^0$C | - |
| $55^0$C | - |
| $65^0$C | - |
| **Growth at pH** | |
| pH 5.0 | + |
| pH 5.7 | + |
| pH 6.8 | + |
| pH 8.0 | + |
| pH 9.0 | + |
| pH 11.0 | + |
| **Growth on NaCl** | |
| 2.5 % | + |
| 5.0% | + |
| 7.0% | + |
| 8.5% | ± |
| 10.0% | - |
| **Growth under anaerobic conditions** | + |
| **Biochemical Tests** | |
| Indole Tests | - |
| Methyl Red test | - |
| Voges Proskauer Test | - |
| Citrate Utilization | + |
| Casein Hydrolysis | - |
| Starch Hydrolysis | - |
| Gelatin Hydrolysis | - |
| Urea Hydrolysis | - |
| Nitrate Reduction | + |
| Nitrite Reduction | - |
| $H_2S$ Production | - |
| Cytochrome oxidase | + |
| **Oxidation/Fermentation/Negative (O/F/-)** | F |
| **Acids production from carbohydrates** | |
| Adonitol | - |
| Arabinose | + |
| Cellobiose | + |
| Dextrose | + |
| Dulcitol | - |
| Fructose | + |
| Galactose | + |
| Inositol | + |
| Inulin | - |
| Lactose | + |
| Maltose | + |
| Mannitol | + |
| Mannose | + |
| Melibiose | + |
| Raffinose | + |
| Rhamnose | + |
| Salicin | + |
| Sorbitol | + |
| Sucrose | + |
| Trehalose | + |
| Xylose | + |

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