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GENETIC CHARACTERIZATION OF *AEROMONAS SP*. BY SUBJECTING RAPD FINGERPRINT PATTERNS TO UPGMA CLUSTER METHOD ANALYSIS

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ABSTRACT

Typing of bacterial pathogens for identifying them at strain level is important for diagnosis, treatment and epidemiological survey of bacterial infections. *Aeromonas sp*. are gram negative pathogenic bacteria of wide spectrum of organisms including humans. Different genetic factors like cytolytic enterotoxin gene AHCYTOEN, aerolysin gene, hemolysin gene have been attributed for its pathogenicity. Characterization of Aeromonads to the species level is difficult due to their phenotypic and genotypic heterogeneity. The aim of this was to use RAPD-PCR fingerprint pattern as a molecular marker and computer assisted pattern analysis to characterize Aeromonas strains. Dendrogram constructed using RAPD-PCR fingerprint patterns demonstrated distinctive clusters at various similarity levels and was helpful in phylogenetic studies of *Aeromonas sp*.

KEYWORDS: RAPD-PCR, Jaccord's Coefficient, UPGMA, NTSYS pc Software, Aeromonas.

INTRODUCTION

*Aeromonas sp*ecies are Gram negative, non-sporous, facultative anaerobic bacteria exhibiting variable shapes from rod to coccoid measuring 0.3mm-3.5mm (Agniswar et al., 2010) and are cosmopolitan in distribution. *Aeromonas sp*ecies are found globally in surface water, ground water, chlorinated drinking water, non-chlorinated 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]. The current taxonomy of the genus Aeromonas is based upon DNA-DNA hybridization and 16S ribosomal DNA related studies. The genera of the family Aeromonadaceae now include *Aeromonas, Oceanimonas, Oceanisphera and Tolumonas* (Incertae sedis) (Martin et al., 2005). Association of Aeromonads with human diseases were reported by (Von Graevenitz and Mensch.,1968) in a review of 30 cases of Aeromonas infection or colonization, providing evidence for their recognition as human pathogens and suggesting that some Aeromonads may be associated with gastrointestinal disease. Today seven species of Aeromonas are recognized to cause a variety of intestinal and extra intestinal infections in humans (Janda and Abbott., 1998). *Aeromonas sp*ecies has been recognized as animal pathogens including frogs and fish (Gosling, 1996) and birds, domestic animals (Gray, 1989; Shane and Gifford, 1985). *Aeromonas sp*p. have been identified as the cause of community acquired infections, nosocomial infection, traveller's diarrhoea (Rautelin et al., 1995) and they have been to cause food borne outbreaks of gastrointestinal disease (Zeng Shan et al., 1988). 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.

The random amplified polymorphic DNA (RAPD) assay, also referred to as arbitrary primed (AP) PCR and DNA amplification fingerprinting (DAF), is a powerful tool for genetic studies. It detects differences along the entire bacterial genome, not only in particular sequences. Thus, this system is helpful in characterizing bacteria isolates over long periodes (Ortiz-Herrera et al., 2004). RAPD can generate various fingerprint profiles with unlimited number of primers (Leal et al., 2004).Typing of bacterial pathogens exhibiting high levels of virulence has applications in studying population dynamics also. Pollard et al., 1990 reported the development of a PCR procedure for rapidly and specifically detecting aerolysin genes in strains of haemolytic oligonucleotide primers. These primers targeted a 209-bp fragment of Aer gene coding for β-haemolytic and detecting template DNA only in PCR. They compared their results with biological assays for haemolytic, cytotoxic and enterotoxic activity. Karunakaran and Devi (1994) reported the production of β-haemolysin by A. caviae which resembled the haemolysin of several other bacteria. They suggested that this activity was regulated due to environmental factors associated with A. caviae in producing haemolysin. Solar et al., 2003 compared three molecular methods (PCR amplification-ERIC, RFLP and REP) for typing A. popoffii isolates independently. Their result showed highest discriminatory tendency to group according to their geographical origin.

Joseph et al., 1979 reported that the presence of plasmids in Aeromonas posed a potential health hazard since plasmids from animals may be transferred to humans either directly, by infection with pathogens or indirectly, if they transferred to humans by way of pathogenic bacteria.

The aim of the present study was to isolate and identify consortium of environmental strains of *Aeromonas sp.* by means of biochemical tests and determine clonal relatedness of strains derived from surface and sub surface soil sediments collected from Forest soil by RAPD-PCR approach.

MATERIAL 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 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 42^0C 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 4^0C.

Bacterial strains and their identification

Nine strains of *Aeromonas sp*p. 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 sp*p. 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 60^0C, 70^0C, 80^0C and 90^0C 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.

(iv) Characterization of DNA by RAPD-PCR technique

The RAPD primers are non-specific and are of small sequences of ten nucleotides only. In the genomic DNA, where ever the complementary sequence of primer is present it gets amplified (Williams, et. al., 1990). The annealing is done at low temperature i. e. 32^0C-35^0C. In the present study eight primers were selected for *Aeromonas sp.* (Ewa, et. al., 2004) and are illustrated in Table 2.The master mix is prepared in a dilution tube for samples of DNA and then is distributed equally in PCR tubes for 30 cycles at RAPD temperature cycle programme. Master mix consisted of reaction mixture (25μl) having 10x buffer, Taq polymerase (1.5U), dNTP's (2mM), Primer (50pM), H_2O and DNA template. The master mix was prepared under aseptic conditions under the laminar air flow and is then subjected to 30 cycles of Initial denaturation at 95^0C for 7 minutes followed by denaturation at 94^0C for 30 sec, Annealing at 32^0C for 40 sec, Extension at 72^0C for 1 min, Final extension at 72^0C for 10 min.

(v) 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, and 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 Aeromonas strains

With the aid of selective media Nine Aeromonas strains (A1 to A9) 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.3). %G+C content of the isolated Aeromonas strains ranged from. 55.3 % to 64.6 %.

RAPD-PCR fingerprinting

The study with RAPD-PCR using Aeromonas caviae genetic material of all the isolates is represented in Fig. 1. The fingerprinting produced multiple DNA products with 05 to 09 bands ranging from 310bp to 1353 bp. The primer produced 05 distinctive PCR products per strain. Maximum number of distinctive primer product was observed in A3 i. e. 09 followed by eight bands in case of A1, A2, A5 and A4 strains, however A6, A7, A8 and A9 showed seven distinctive products. The bands in each of the patterns produced by RAPD method were analyzed by Jaccord's coefficient. The numerical analysis of RAPD profiles revealed 03 distinctive clusters, which shows genetic relatedness Fig. 2. First cluster consists of strain A3 that differs with another group of A5 at 86% similarity level. Closely related strains including A1, A2, A4, A9, A8 fall within same similarity group. However, strain A7 shows similar pattern of RAPD fingerprinting and separate from A1, A2, A4, A9 and A8 at 88% similarity. The clusters fall separates with only A6 strain which shows polymorphic bands beginning from the similarity level as obtained from NTSYS distribution pattern.

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

Table2. Primers used in RAPD analysis of *Aeromonas sp.*

S. No.	Primer Name	Primer Sequence
01.	OPB-1	5'-GTTTCGCTCC-3'
02.	OPB-6	5'-TGCTCTGCCC-3'
03.	OPB-7	5'-GGTGACGCAG-3'
04.	AP3	5'-TCACGATGCA-3'
05.	AP5	5'-TCACGCTGCG-3'
06.	AK1	5'-ATCACTATGA-3'
07.	AK2	5'-GATCCTGCAG-3'
08.	AK3	5'-TAAGGTTCGG-3'

Table3. Biochemical tests as displayed by Aeromonas strains

S. No.	Biochemical Test	Result
1.	Colony Morphology	
	Configuration	Round
	Margin	Entire
	Elevations	Convex
	Surface	Smooth
	Density	Translucent
	Pigments	-ve
	Gram's Reaction	-ve
	Cell shape	Rods
	Size	Short
	Arrangement	Single
2.	**Characteristics**	
	Spore(s)	-
	Capsule	-
	Motility	+
	Fluorescence (UV)	-
3.	**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	-
4.	**Growth at pH**	
	pH 5.0	+
	pH 5.7	+
	pH 6.8	+
	pH 8.0	+
	pH 9.0	+
	pH 11.0	+
5.	**Growth on NaCl**	
	2.5 %	+
	5.0%	+
	7.0%	+
	8.5%	±
	10.0%	-
6.	**Growth under**	+

	anaerobic conditions	
7.	**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	+
8.	**Oxidation/Fermentation/Negative (O/F/-)**	F
9.	**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	+

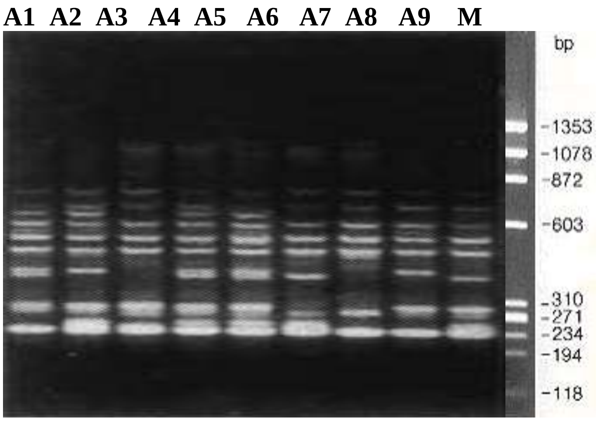

Fig. 1 RAPD PCR of *Aeromonas sp*. isolated from forest soil of Raisen district with primer AP5.
M – Marker
A1 to A5 – surface soil isolate
A6 to A9 - deep soil isolate

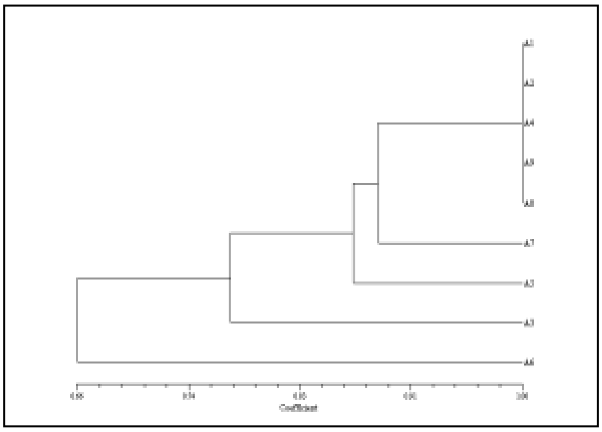

Fig.2 Dendrogram showing genetic relatedness of 09 strains of *Aeromonas sp.* from forest soil of Raisen District determined by analysis of RAPD-PCR fingerprint patterns using Jaccord's similarity Coefficient and UPGMA cluster method.

DISCUSSIONS

The interrelationships of microbial strains from various habitats can be studied by analyzing their genetic fingerprint. RAPD-PCR is a method for genetic fingerprinting by random amplification of polymorphic DNA (Williams et al., 1990 and Welsch et al., 1990). This technique has been employed for genomic characterization owing to its simplicity, reproducibility rapidity and discriminatory power. Lunge,et. al., 1994 for e.g. analyzed genomic DNA from *Bradyrhizobium japonicum* strain by Random amplified polymorphic DNA (RAPD). Polymorphism was observed in RAPD fingerprint. The RAPD banding patterns of different strains were used to calculate the genetic divergence and to construct phylogenetic trees allowing studies of the relationship between the strains. RAPD separated the *Bradyrhizobium* strains into four main groups. RAPD allowed closely related strains to be distinguished. The RAPD method appears to have numerous applications for characterizing such microbial populations (Hardy's, et. al., 1992).

Miyata et al., 1995 used RAPD technique to analyze the genetic differentiation of 13 strains of *A.salmonicida* and 7 strains of *A.hydrophila*. They found that RAPD profiles of all non motile aeromonads differed between isolates revealing genomic homogeneity in A. salmonicida subsp. salmonicida and genetic variability in *A.hydrophila* strains. Oakey et al., 1996 investigated the differentiation of the genus Aeromonas at genomospecies level using RAPD-PCR techniques. Of the 20 primers evaluated, they found that 6 produced profiles which contained multiple bands capable of differentiating the genomospecies thus, illustrating genetic heterogeinity. Also they reported that this study supported the current classification of Aeromonas into genospecies. Oakey et al., 1999 investigated A. hydrophila (HG 1)-specific RAPD-PCR fragments for their potential as DNA probes. They found that RAPD-PCR generated fragments 7e3, 7e5 and 7e6 were specific to A. hydrophila (HG-1) isolates and the sequences of these fragments could be used to design HG-1 specific primers. They achieved the specificity of the PCR by using modified hot-start procedure. O'hIci et al., 2000 reported the genotypic analysis of 39 strains of typical A. salmonicida subsp. All the strains were analyzed using RAPD and PFGE techniques and the genetic similarities were evaluated. Their results showed that A. salmonicida constituted a genetically heterogeneous group of strains and there was very less correlation between the two methods in identifying clusters of similar atypical strains.

Delamare et al., 2002 compared the efficiency of RAPD and SDS-PAGE methods for the differentiation of *Aeromonas sp*p. And their results showed that SDS-PAGE had the potential to differentiate *Aeromonas sp*p. But its low qualitative variation indicated that this technique was not efficient for characterization of strains within species, while RAPD allowed the identification of strains but the high variability limited its potential as an aiding method for species identification.

CONCLUSIONS

Above data indicates that modern methods like PCR etc. are effective to differentiate virulent and avirulent strains of Aeromonas. RAPD-PCR method had the discriminatory power and has proved to be useful for epidemiological investigation and population genetic analysis of *Aeromonas sp*p. Different combinations of the virulence genes present in the isolates indicate their probable role in the pathogenesis of Aeromonas infections. Therefore, in future, specific probes pertaining to different virulence genes could be developed for correct identification and characterization of pathovars of *Aeromonas sp*. This study emphasizes the importance of using more than one method for the correct identification of Aeromonas strains like ERIC, BOX-PCR etc. In conclusion, RAPD-PCR has the benefit of being quicker, easier and required less sophisticated instrumentation for genetic characterization of *Aeromonas sp*. and primer AP5 was well suited for epidemiological differentiation and cluster analysis of *Aeromonas sp*.

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