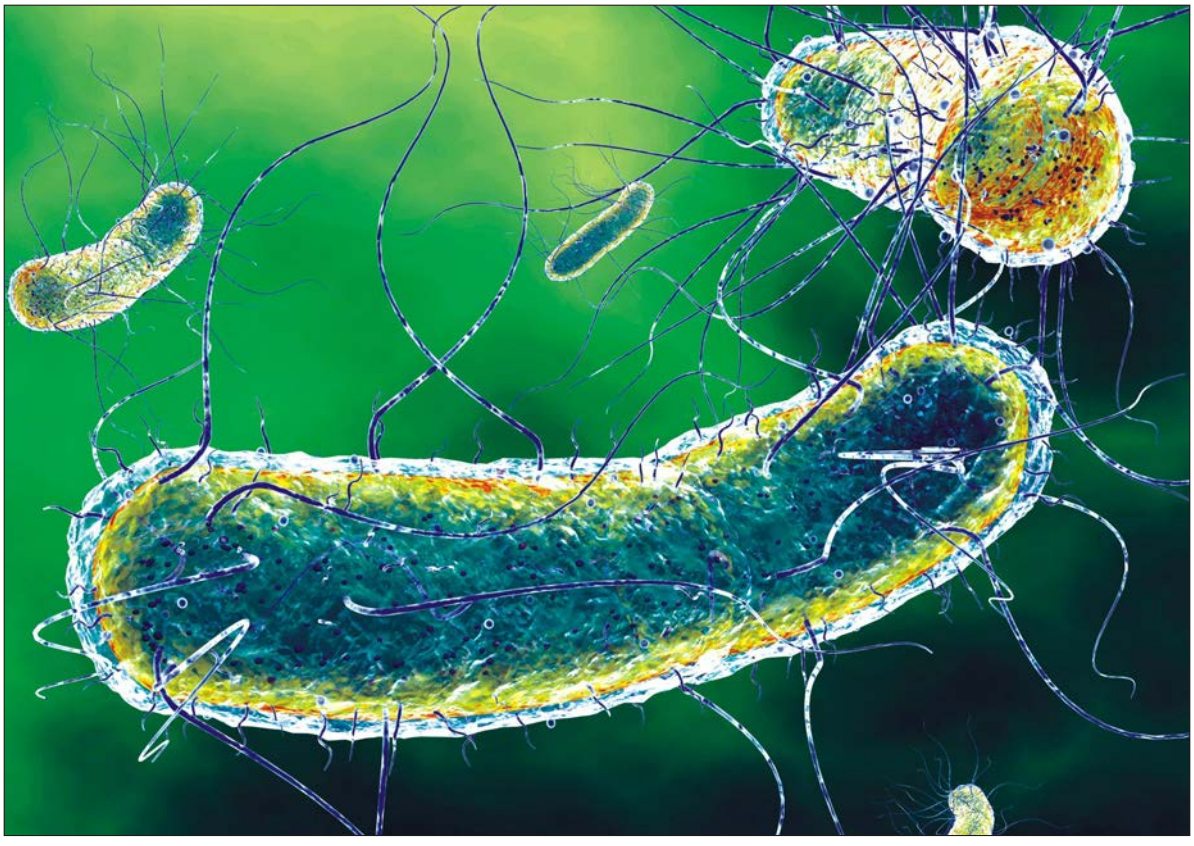

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Antibiotic Susceptibility and Genetic Analysis of *Vibrio* Species Isolated from Reverine Environment

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Abstract: The resistance profile and its correlation with mobile genetic elements were investigated in 11 *Vibrio cholerae,* 10 *V. parahaemolyticus*, 12 *V. vulnificus,* 11 *V. fischeri,* 10 *V. proteolyticus* and 5 *V. mimicus* isolated from River Narmada. All the 59 isolates of *Vibrio* species were examined for their susceptibility/resistance against 14 commonly used antibiotics against *Vibrio* species. More than 50% isolates showed resistance against five commonly used antibiotics viz., ampicillin, ceftadizime, erythromycin, chloramphenicol, cefuroxime. Plasmid of 6 kb was detected in 11 resistant isolates and class 1 integron was detected in 16 resistant isolates. SXT element was not found among resistant isolates. The present study indicated that plasmid and Class 1 integron mainly contributed to the circulation of multidrug resistance determinants in *Vibrio* species isolated from river Narmada.

Key words: Class I integron, multidrug resistance, plasmid, SXT element, *Vibrio*

INTRODUCTION

The genus *Vibrio* includes many harmless species, native of fresh, brackish and marine water (Colwell *et al*., 1994; Sharma and Chaturvedi, 2007). However from reverine system, the serotypes O1 and O139 of *Vibrio cholerae* and some *V. cholerae* non O1 and O139 serotypes (Sharma and Chaturvedi, 2004, 2006; Gil *et al*., 2004), *V. parahaemolyticus* (Utsalo *et al*., 1992), *V. fluvialis* (Ahmed *et al*., 2004) and *V. aliginolyticus* (Ripabelli *et al*., 2003) have been documented as etiological agents of enteric diseases and epidemics.

Infections caused by these organisms are usually associated with ingestion of raw shellfish or exposure of wound to seawater. The clinical presentation and severity of infections are wide ranging. The most common presentation is self limiting gastroenteritis, but soft tissue infection and septicemia do occur and their morbidity and mortality are high, particularly in patient with liver disease (Rowe-Magnus *et al*., 2006). The pathogenicity of cholera, caused by *Vibrio cholerae* O1 and O139 strains, food poisoning caused by *V. parahaemolyticus* and septicemia caused by *V. vulnificus* has governed the most attention and these species have been documented as human pathogens. The diarrhoeal symptoms caused by *V. cholerae* and *V. parahaemolyticus* include profuse purging of watery stool, vomiting and dehydration. *Vibrio hollisae*, *V. fluvialis* and *V. fetus* have also been associated with human diseases (Abott and Janda, 1994; Ahmed *et al*., 2004).

Antimicrobial resistance has become a major medical and public health problem as it has direct link with disease management (Ramamurthy, 2008). Antibiotics such as tetracycline,

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doxycycline, norfloxacin, ciprofloxacin, streptomycin and fluoroquinolones may be used as an adjunet to rehydration therapy and are critical in the treatment of septicemia patients (Lima, 2001; Bhattacharya, 2003; Chiang and Chuang, 2003). Resistance to many of these drugs which have emerged in these pathogens is a matter of major concern, particularly in case of *V. vulnificus* and *V. cholerae* (Rowe-Magnus *et al*., 2006). Multiple antibiotic resistance genes clustered within the same genetic locus (Resistance Island) can be transferred to other organisms. Spread of antibiotic resistance in microbes has been attributed to the mobilization of drug resistance markers by a variety of agents like plasmid, transposons and integrons (Olsen, 1999; White and McDermott, 2001). In *Vibrio* sp. antibiotic resistance determinants have been traditionally found on plasmid. Recently, in few cases these determinants have also been detected on integrons and a novel conjugative transposable element i.e., SXT (Amita *et al.*, 2003).

River Narmada is the largest west flowing river of the Indian subcontinent and the fifth largest river of Indian peninsula. Among all the sacred rivers of India, the Narmada occupies a unique place. It originates from Maikala ranges at Amarkantak in Madhya Pradesh at an elevation of 1065 m. The water quality of the river is being influenced by the inputs derived from land use and human activities in the surrounding region from non-point sources. These land uses include agriculture, animal farming and residential development. The study and evaluation of mobile genetic elements will provide an indepth knowledge regarding the presence of gene cassettes and integrons responsible for the spread of multidrug resistance in *Vibrio* gene pool in reverine system.

MATERIALS AND METHODS

Isolation of *Vibrio* sp. (Kaper *et al.*, 1979)

Water samples were collected from two stations i.e., Amarkantak and Hoshangabad. water samples (1000 mL, v/v) were collected aseptically, concentrated on 0.45 μm pore diameter filter and enriched in alkaline peptone water (1% peptone, 1% NaCl, pH 9) for isolation of *Vibrio* sp. Bacterial colonies were isolated from the enrichment cultures by using Thiosulfate-Citrate-Bile Salt (TCBS) agar medium and incubating it for 24 h at 37°C. A total of 59 environmental isolates of *Vibrio* sp. were used in the present study.

Biochemical Identification of *Vibrio* sp. from River Narmada (Alsina and Blanch, 1994)

All the isolates were examined for the following characteristics: Arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, halophilism test (0, 3, 6, 8 and 10% NaCl), aesculin hydrolysis, citrate, gelatinase, indole, oxidase, urease, voges proskauer, acid from arabinose, inositol, mannitol, sorbitol and sucrose were employed. The isolates were identified with the help of Bergey's Manual of Systematic Bacteriology (Krieg and Holt, 1984) and PIB (Probabilistic Identification of Bacteria) computer kit (Bryant, 1993). The isolates were deposited in Bacterial Germplasm Culture Collection, R.D. University, Jabalpur (MP), India and were given BGCC numbers.

Molecular Differentiation of *Vibrio* sp.

Isolation of Genomic DNA

Genomic DNA was extracted following a modified scheme of Murray and Thompson (1980). Cells from an 18 h old culture grown in Luria Bertani broth were collected and resuspended in 567 μL of Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA pH 8.0), treated with 10% (w/v) sodium dodecyl sulphate and freshly prepared proteinase K (0.1 mg mL^{-1})

and incubated at 37°C for 1 h. After incubation, 10% cetyl trimethyl ammonium bromide in 0.7 M NaCl was added (10 mg mL^{-1}) and incubated at 65°C for 10 min. The DNA was extracted with phenol: chloroform: iso amyl alcohol (25:24:1). The aqueous phase was transferred to a fresh micro centrifuge tube and DNA was precipitated with an equal volume of isopropanol. Pellet was washed with 70% ethanol and dried. The concentration of DNA was then determined spectrophotometrically (1 OD_{260}= 50 µg of double stranded DNA mL^{-1}).

Amplified Ribosomal DNA Restriction Analysis (Urakawa *et al*., 1997)

The primer selected had the following sequence F: 5'-GCCTAACACATGCAAGTCGA-3' and R 5'- CGTATTACCGCGGCTGCTGG-3'. These primers target two highly conserved regions of the prokaryotic 16S rRNA gene that flank a number of regions known to be variable among bacterial species. Each 25 µL reaction contained 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 2.0 mM $MgCl_2$, 100 mM concentration of each deoxynucleotides triphosphate, 400 mM concentration of each primer and 1 U of Taq polymerase. Polymerase chain reaction amplification was performed using a gene amplification system (Corbett research CG1-96). The initial cycle consisted of 3 min at 94°C, 30 sec at 57°C and 30 sec at 72°C and was followed by 30 cycles of 94°C for 30 sec, 57°C for 30 sec and 72°C for 30 sec. The final cycle consisted of 94°C for 30 sec and 57°C for 30 sec and an extension at 72°C for 10 min. The 16S rDNA type was determined by digestion of the amplicons with AluI (1U) and HaeIII (1U). Each 20 µL reaction contained 10 µL of PCR product, 1.5 µL of each enzyme (1U), 1.5 µL buffer (1X), 7 µL double distilled water The reaction mixture was incubated on a water bath at 37°C for 12 h. The restriction pattern was analyzed on a 2% agarose gel staining with ethidium bromide and visualized under an UV transilluminator.

Random Amplified Polymorphic DNA Analysis (Arias *et al*., 1998)

The RAPD was performed with a gene amplification system (Corbett research CG1-96). The primer selected had the following sequence: 5'- GCGATCCCCA-3'. Each 25.0 µL of the RAPD reaction mixture contained the following reagents: 2.5 µL of 10X reaction buffer 500 mM Kcl, 100 mM Tris HCl (pH 8.3), 2.5 µL of a solution containing each of the deoxynucleoside triphosphates at concentration of 2.5 mM, 2.5 µL of 25 mM $MgCl_2$, 20 pmol of primer, 1U of r Taq DNA polymerase and 3.0 µL of template. The reaction volume was adjusted to 25 µL using sterile double distilled water. The reaction mixtures were subjected to initial denaturation at 94°C for 5 min, followed by 45 cycles of 94°C for 1 min, 36°C for 1 min and 72°C for 2 min. and a final extension step at 72°C for 5 min. The amplified products were electrophoresed on 1.5% agarose gel staining containing ethidium bromide and visualized under an UV transilluminator.

Statistical Analysis

ARDRA and RAPD-PCR bands were scored as either present (1) or absent (0). All binary data were entered and genetic distances were calculated through Numerical Taxonomy and Multivariate Analysis System (NTSYS-pc), version 2.02. The Euclidean distance was determined and then dendrogram was assembled using Unweighted Paired Group Method using Arithmetic average criterion (UPGMA).

Antibiotic Susceptibility Test (Bauer *et al*., 1966)

Characterization of the resistance/susceptibility profile of the isolates was determined by disc diffusion method on Mueller-Hinton agar using commercially available antibiotic-

impregnated discs (Hi-Media Laboratories Mumbai, India) containing ampicillin (10 mcg), co-trimoxazole (1.25 mcg), amikacin (30 mcg), cefuroxime (30 mcg), erythromycin (15 mcg), doxycyline hydrochloride (30 mcg), streptomycin (300 mcg), chloramphenicol (10 mcg), ciprofloxacin (5 mcg), ceftazidime (30 mcg), nalidixic acid (30 mcg), tetracycline (30 mcg), trimethoprim (30 mcg) and norfloxacin (10 mcg). Plates containing antibiotic discs for each bacterial strain were incubated at 37°C for 24 h. The bacterial isolates were recorded either as resistant or sensitive by measuring inhibitory zone and comparing with the interpretative chart.

Plasmid Profiling

The presence of plasmid was examined by alkaline lysis method of Sambrook *et al*. (1989) with certain modifications. Bacterial cells were grown in 10 mL Luria Bertani broth and incubated overnight at 37°C. The pellet was collected by centrifugation at 5000 rpm for 15 min and dried. The cells were lysed by adding 100 µL of solution I (50 mM glucose, 25 mM Tris HCl; pH 8.0, 10 mM EDTA; pH 8.0) containing 1 µg lysozyme mL^{-1} followed by incubation on ice for 15 min 200 µL of solution II (0.2 N NaOH and 1% SDS) was added and gently mixed. 150 µL ice cold solution III (5 M potassium acetate, glacial acetic acid 11.5 mL and distilled water 28.5 mL) was added subsequently and mixture was centrifuged at 13000 rpm for 15 min. One milliliter of chilled ethanol was added to the supernatant and incubated on ice for 45 min followed by centrifugation at 13000 rpm for 15 min.

The pellet was resuspended in 100 µL of TE buffer containing 1 µg of RNase and kept at 37°C in water bath for 30 min. Twenty microliter of 3 M sodium acetate and 770 µL chilled ethanol was added to above solution and kept on ice for 1 h. The plasmid DNA was collected by centrifugation at 13000 rpm for 15 min. The pellet was dissolved in 50 µL TE buffer (10 mM). Ten microliter of plasmid DNA was electrophoresed on 0.7% agarose gel (Bangalore Genei, India) containing ethidium bromide (0.5 mg L^{-1}) at 100 v in 0.5X TAE buffer (0.4 M Tris HCl, 0.02 M $Na_2EDTA.2H_2O$, 0.2 M sodium acetate, 1.02 M acetic acid) and visualized under an UV transilluminator.

Detection of Class 1 Integron (Miyazoto *et al*., 2004)

Integrons are natural genetic engineering platform that incorporate Open Reading Frames (ORFs) and convert them into functional genes by ensuring their correct expression (Recchia and Hall, 1995). To identify class 1 integron, primers in DS-F (5'- CGGAATGGCCGAGCAGATC-3') and DS-B (5'-CAAGGTTTGGACCAGTTGCA-3'), which are specific for the 5' conserved segment containing integrase gene (intI 1) were used. The PCR (Corbett research CG1-96) amplification included initial denaturation at 94°C for 3 min for 1 cycle, 35 thermal cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 2 min 30 sec for 35 cycles with final extension of 5 min at 72°C for 1 cycle. Polymerase chain reaction products were concentrated by electrophoresis on 1% agarose gel (Bangalore Genei, India) containing ethidium bromide (0.5 mg L^{-1}) at 100 volt in 0.5X TAE buffer (0.4 M Tris HCl, 0.02 M Na_2 EDTA. $2H_2O$, 0.2 M sodium acetate, 1.02 M acetic acid) and visualized under an UV transilluminator.

Detection of SXT Element (Waldor *et al*., 1996)

To detect the presence of SXT element in the genome, the Primer sequences INT 1 (5'-GCTGGATAGGTTAGGGCGG-3') and INT 2 (5'-CTCTATGGGCACTGTCCACATTG-3') specific for SXT integrase gene (int_{SXT}) were used. PCR (Corbett research CG1-96) conditions were as follows: 94°C for 5 min followed by 35 cycles of 94°C for 30 sec, 60°C for 1 min and

72°C for 2 min, followed by final extension at 72°C for 10 min. Polymerase chain reaction products were electrophoresed on 1% agarose gel (Bangalore Genei, India) containing ethidium bromide (0.5 mg L^{-1}) at 100 Volt in 0.5X TAE buffer (0.4 M Tris HCl, 0.02 M $Na_2EDTA.2H_2O$, 0.2 M sodium acetate, 1.02 M acetic acid) and visualized under an UV transilluminator.

RESULTS

The molecular diversity and evolution of groups of aquatic bacteria deserve an in depth investigation, so as to understand, their ecological role and function in aquatic environment. In the present investigation 59 *Vibrio* strains were isolated from surface and subsurface water samples collected from Amarkantak and Hoshangabad stations of River Narmada. 32 strains were isolated from four sites (Udgamkund, Mai ki Bagiya, Kapildhara, Stop dam region) of Amarkantak and 27 strains were isolated from four sites (Sethani Ghat, Pichin Ghat, Mangalwara Ghat, Muktidham Ghat) of Hoshangabad. Fifty nine isolates were distributed between six different species identified on basis of biochemical tests (Table 1).

The ARDRA analysis of 59 isolates showed bands ranging from 2 to 7 with molecular weight between 300 bp to 1200 bp. 10 isolates of *V. parahaemolyticus* showed 4 bands having molecular weight 700 , 850, 1000 and 1200 bp. 11 isolates of *V. cholerae* showed 7 bands with molecular weight of 275, 300, 325, 400, 500, 600 and 700 bp. 10 isolates of *V. proteolyticus* showed 2 bands having molecular weight of 850 and 1050 bp. 11 isolates of *V. fischeri* showed 3 bands with molecular weight of 850, 900 and 1200 bp. 12 *isolates* of *V. vulnificus* showed 5 bands having molecular weight 300, 500, 600, 900 and 1500 bp and 5 isolates of *V. mimicus* showed 3 bands with molecular weight of 650, 400 and 1050 bp (Fig. 1). The UPGMA cluster analysis of ARDRA profile showed 100% similarity at a coefficient of 2.47. At 1.92 coefficient, two major clusters were formed (A and B). The cluster A was subdivided into two sub divisions (A1 and A2) at 1.736 coefficient level, consisting of *V. proteolyticus* and *V. fischeri*. Similarly, cluster B was subdivided into two subdivisions (B1 and B2) at 1.92 coefficient level that included species *V. cholerae*, *V. mimicus*, *V. parahaemolyticus*, *V. vulnificus* (Fig. 2).

The RAPD analysis of the *Vibrio* spp. showed 4 to 5 bands with molecular weight between 110 bp to 1000 bp. 10 isolates of *V. parahaemolyticus* and 11 isolates of *V. cholerae* showed 4 bands having molecular weight 150, 200, 400 and 500 bp. Eleven isolates of *V. fischeri* showed 4 bands having molecular weight 150, 250, 475 and 600 bp. 12 *isolates* of

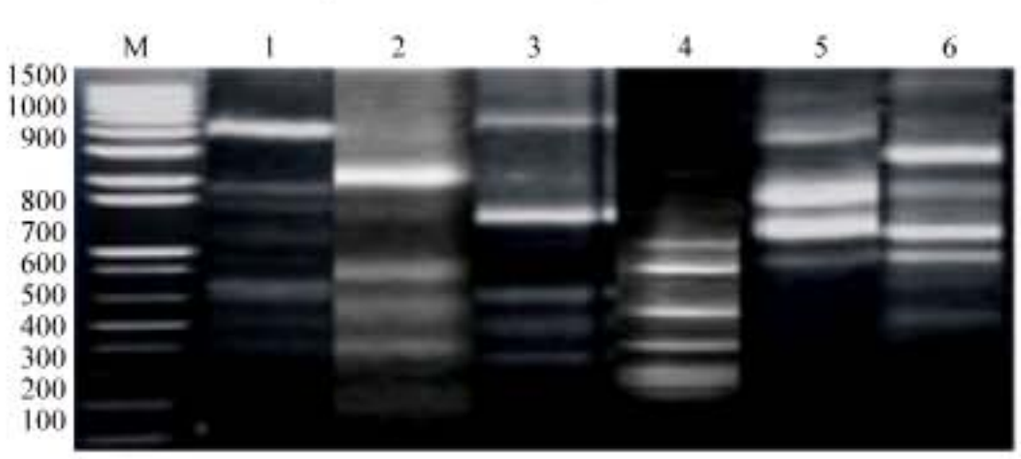

Fig. 1: Representative gel of ARDRA profile, M: Marker, lane 1: *V. parahaemolyticus* (BGCC# 673-682), 2: *V. fischeri* (BGCC#695-705), 3: *V. mimicus* (BGCC# 716-720), 4: *V. cholerae* (BGCC# 662-672) 5: *V. proteolyticus* (BGCC# 706-715), 6: *V. vulnificus* (BGCC# 683-694)

Table 1: Identification of *Vibrio* sp. isolated from river Narmada based on biochemical characteristics

	Identification parameters																						
Isolate No	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	Identified species
BGCC662	-	-	+	+	+	+	-	-	-	-	+	+	-	+	-	+	-	-	+	-	-	+	*V. cholerae*
BGCC663	-	-	+	+	+	+	-	-	-	-	+	+	-	+	-	+	-	-	+	-	-	+	*V. cholerae*
BGCC664	-	-	+	+	+	+	-	-	-	-	+	+	-	+	-	+	-	-	+	-	-	+	*V. cholerae*
BGCC665	-	-	+	+	+	+	-	-	-	-	+	+	-	+	-	+	-	-	+	-	-	+	*V. cholerae*
BGCC666	-	-	+	+	+	+	-	-	-	-	+	+	-	+	-	+	-	-	+	-	-	+	*V. cholerae*
BGCC667	-	-	+	+	+	+	-	-	-	-	+	+	-	+	-	+	-	-	+	-	-	+	*V. cholerae*
BGCC668	-	-	+	+	+	+	-	-	-	-	+	+	-	+	-	+	-	-	+	-	-	+	*V. cholerae*
BGCC669	-	-	+	+	+	+	-	-	-	-	+	+	-	+	-	+	-	-	+	-	-	+	*V. cholerae*
BGCC670	-	-	+	+	+	+	-	-	-	-	+	+	-	+	-	+	-	-	+	-	-	+	*V. cholerae*
BGCC671	-	-	+	+	+	+	-	-	-	-	+	+	-	+	-	+	-	-	+	-	-	+	*V. cholerae*
BGCC672	-	-	+	+	+	+	-	-	-	-	+	+	-	+	-	+	-	-	+	-	-	+	*V. cholerae*
BGCC673	-	-	-	-	-	+	+	+	+	-	-	-	-	+	-	-	+	-	-	-	-	-	*V. parahaemolyticus*
BGCC674	-	-	-	-	-	+	+	+	+	-	-	-	-	+	-	-	+	-	-	-	-	-	*V. parahaemolyticus*
BGCC675	-	-	-	-	-	+	+	+	+	-	-	-	-	+	-	-	+	-	-	-	-	-	*V. parahaemolyticus*
BGCC676	-	-	-	-	-	+	+	+	+	-	-	-	-	+	-	-	+	-	-	-	-	-	*V. parahaemolyticus*
BGCC677	-	-	-	-	-	+	+	+	+	-	-	-	-	+	-	-	+	-	-	-	-	-	*V. parahaemolyticus*
BGCC678	-	-	-	-	-	+	+	+	+	-	-	-	-	+	-	-	+	-	-	-	-	-	*V. parahaemolyticus*
BGCC679	-	-	-	-	-	+	+	+	+	-	-	-	-	+	-	-	+	-	-	-	-	-	*V. parahaemolyticus*
BGCC680	-	-	-	-	-	+	+	+	+	-	-	-	-	+	-	-	+	-	-	-	-	-	*V. parahaemolyticus*
BGCC681	-	-	-	-	-	+	+	+	+	-	-	-	-	+	-	-	+	-	-	-	-	-	*V. parahaemolyticus*
BGCC682	-	-	-	-	-	+	+	+	+	-	-	-	-	+	-	-	+	-	-	-	-	-	*V. parahaemolyticus*
BGCC683	-	-	+	+	-	+	+	+	-	-	+	+	+	+	+	-	+	-	+	-	-	-	*V. vulnificus*
BGCC684	-	-	+	+	-	+	+	+	-	-	+	+	+	+	+	-	+	-	+	-	-	-	*V. vulnificus*
BGCC685	-	-	+	+	-	+	+	+	-	-	+	+	+	+	+	-	+	-	+	-	-	-	*V. vulnificus*
BGCC686	-	-	+	+	-	+	+	+	-	-	+	+	+	+	+	-	+	-	+	-	-	-	*V. vulnificus*
BGCC687	-	-	+	+	-	+	+	+	-	-	+	+	+	+	+	-	+	-	+	-	-	-	*V. vulnificus*
BGCC688	-	-	+	+	-	+	+	+	-	-	+	+	+	+	+	-	+	-	+	-	-	-	*V. vulnificus*
BGCC689	-	-	+	+	-	+	+	+	-	-	+	+	+	+	+	-	+	-	+	-	-	-	*V. vulnificus*
BGCC690	-	-	+	+	-	+	+	+	-	-	+	+	+	+	+	-	+	-	+	-	-	-	*V. vulnificus*
BGCC691	-	-	+	+	-	+	+	+	-	-	+	+	+	+	+	-	+	-	+	-	-	-	*V. vulnificus*
BGCC692	-	-	+	+	-	+	+	+	-	-	+	+	+	+	+	-	+	-	+	-	-	-	*V. vulnificus*
BGCC693	-	-	+	+	-	+	+	+	-	-	+	+	+	+	+	-	+	-	+	-	-	-	*V. vulnificus*
BGCC694	-	-	+	+	-	+	+	+	-	-	+	+	+	+	+	-	+	-	+	-	-	-	*V. vulnificus*
BGCC695	-	-	+	+	-	+	+	-	-	-	+	-	-	+	+	-	-	-	+	+	-	+	*V. fischeri*
BGCC696	-	-	+	+	-	+	+	-	-	-	+	-	-	+	+	-	-	-	+	+	-	+	*V. fischeri*
BGCC697	-	-	+	+	-	+	+	-	-	-	+	-	-	+	+	-	-	-	+	+	-	+	*V. fischeri*
BGCC698	-	-	+	+	-	+	+	-	-	-	+	-	-	+	+	-	-	-	+	+	-	+	*V. fischeri*
BGCC699	-	-	+	+	-	+	+	-	-	-	+	-	-	+	+	-	-	-	+	+	-	+	*V. fischeri*
BGCC700	-	-	+	+	-	+	+	-	-	-	+	-	-	+	+	-	-	-	+	+	-	+	*V. fischeri*
BGCC701	-	-	+	+	-	+	+	-	-	-	+	-	-	+	+	-	-	-	+	+	-	+	*V. fischeri*
BGCC702	-	-	+	+	-	+	+	-	-	-	+	-	-	+	+	-	-	-	+	+	-	+	*V. fischeri*
BGCC703	-	-	+	+	-	+	+	-	-	-	+	-	-	+	+	-	-	-	+	+	-	+	*V. fischeri*
BGCC704	-	-	+	+	-	+	+	-	-	-	+	-	-	+	+	-	-	-	+	+	-	+	*V. fischeri*
BGCC705	-	-	+	+	-	+	+	-	-	-	+	-	-	+	+	-	-	-	+	+	-	+	*V. fischeri*
BGCC706	-	-	+	+	-	+	+	-	-	-	+	+	+	+	-	-	-	-	+	+	-	+	*V. proteolyticus*
BGCC707	-	-	+	+	-	+	+	-	-	-	+	+	+	+	-	-	-	-	+	+	-	+	*V. proteolyticus*
BGCC708	-	-	+	+	-	+	+	-	-	-	+	+	+	+	-	-	-	-	+	+	-	+	*V. proteolyticus*
BGCC709	-	-	+	+	-	+	+	-	-	-	+	+	+	+	-	-	-	-	+	+	-	+	*V. proteolyticus*
BGCC710	-	-	+	+	-	+	+	-	-	-	+	+	+	+	-	-	-	-	+	+	-	+	*V. proteolyticus*
BGCC711	-	-	+	+	-	+	+	-	-	-	+	+	+	+	-	-	-	-	+	+	-	+	*V. proteolyticus*
BGCC712	-	-	+	+	-	+	+	-	-	-	+	+	+	+	-	-	-	-	+	+	-	+	*V. proteolyticus*
BGCC713	-	-	+	+	-	+	+	-	-	-	+	+	+	+	-	-	-	-	+	+	-	+	*V. proteolyticus*
BGCC714	-	-	+	+	-	+	+	-	-	-	+	+	+	+	-	-	-	-	+	+	-	+	*V. proteolyticus*
BGCC715	-	-	+	+	-	+	+	-	-	-	+	+	+	+	-	-	-	-	+	+	-	+	*V. proteolyticus*
BGCC716	-	+	+	-	-	+	+	+	+	-	+	+	+	+	+	+	-	-	-	-	+	-	*V. mimicus*
BGCC717	-	+	+	-	-	+	+	+	+	-	+	+	+	+	+	+	-	-	-	-	+	-	*V. mimicus*
BGCC718	-	+	+	-	-	+	+	+	+	-	+	+	+	+	+	+	-	-	-	-	+	-	*V. mimicus*
BGCC719	-	+	+	-	-	+	+	+	+	-	+	+	+	+	+	+	-	-	-	-	+	-	*V. mimicus*
BGCC720	-	+	+	-	-	+	+	+	+	-	+	+	+	+	+	+	-	-	-	-	+	-	*V. mimicus*

1: Gram reaction; 2: Arginine dihydrolase; 3: Lysine decaroxylase; 4: Ornithine decaroxylase; 5: Growth at 0% NaCl; 6: Growth at 3% NaCl; 7: Growth at 6% NaCl; 8: Growth at 8% NaCl; 9: Growth at 10% NaCl; 10: Aesculin hydrolysis; 11: Citrate utilization; 12: Gelatinase; 13: Indole; 14: Oxidase; 15: Urease; 16: VP; 17: Acid from Arabinose; 18: Acid from Inositol; 19: Acid from Mannitol; 20: Acid from Salicin; 21: Acid from Sorbitol; 22: Acid from Sucrose, +: Positive reaction, -: Negative reaction, BGCC: Bacterial germplasm collection center

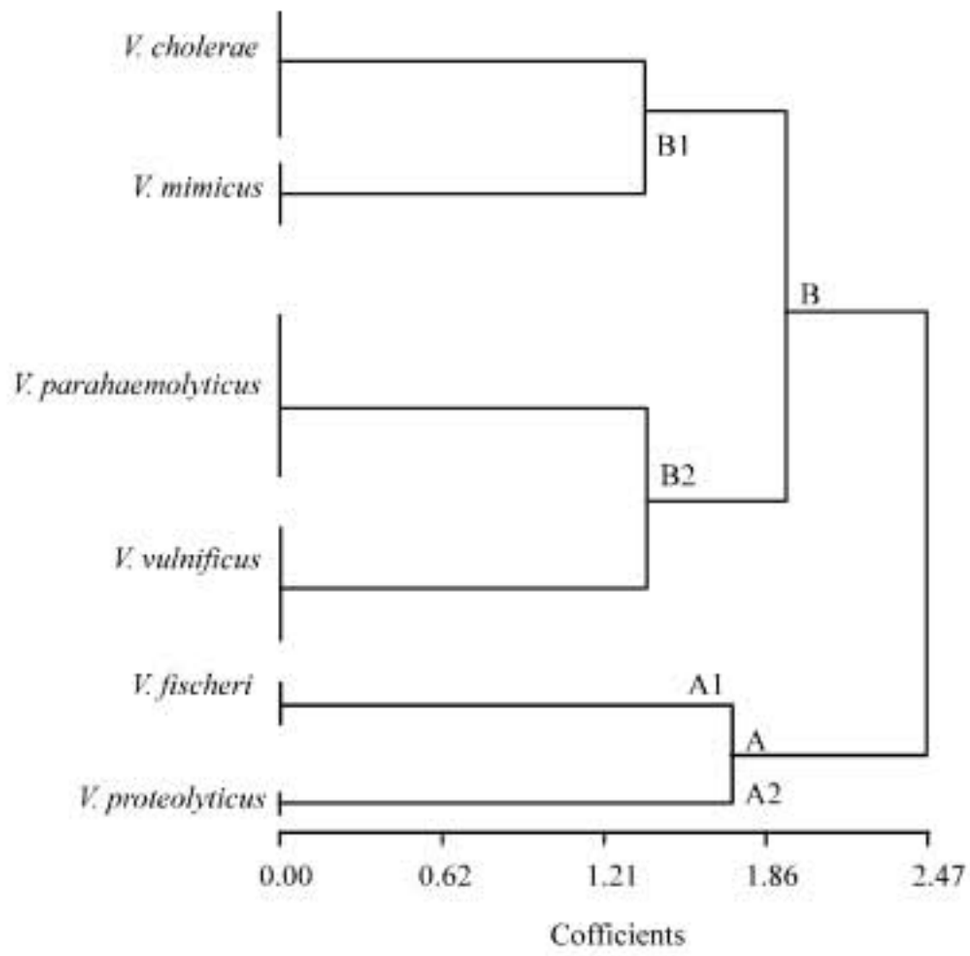

Fig. 2: UPGMA cluster analysis on the basis of ARDRA of *Vibrio* isolated from River Narmada

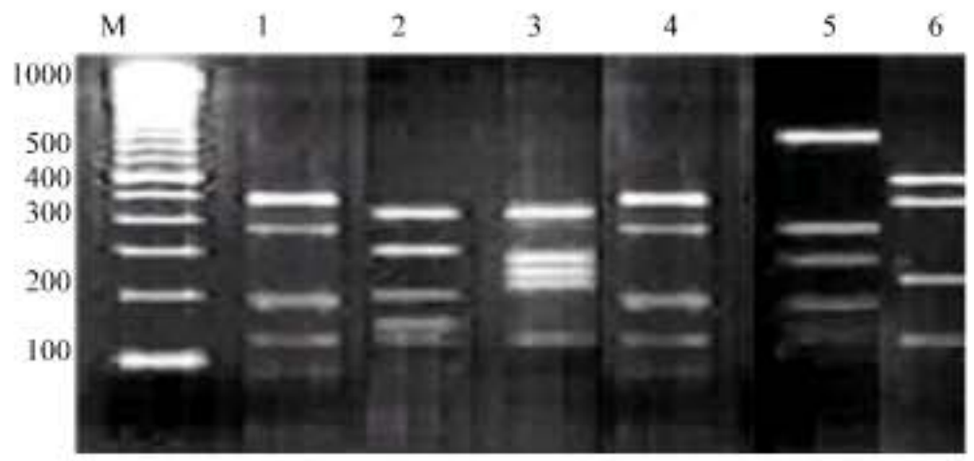

Fig. 3: Representative gel of RAPD profile, M: Marker, lane 1: *V. parahaemolyticus* (BGCC# 673-682), 2: *V. proteolyticus* (BGCC#706-715), 3: *V. mimicus* (BGCC# 716-720), 4: *V. cholerae* (BGCC# 662-672) 5: *V. fischeri* (BGCC#695-705, 6: *V. vulnificus* (BGCC#683-694)

V. vulnificus showed 4 bands having molecular weight 175, 275, 310 and 1000 bp. 10 isolates of *V. proteolyticus* showed 5 bands having molecular weight 110, 150, 200, 300 and 475 bp. 5 isolates of *V. mimicus* showed 5 bands having molecular weight 150, 210, 250, 275 and 475 bp (Fig. 3). UPGMA cluster analysis of RAPD profile showed 100% similarity at a coefficient of 1.42. At 2.84 Coefficient, two major clusters were formed (A and B). The cluster B sub divisions (B1, B2 and B3) at 2.13 coefficient level, consisting of species *V. fischeri*, *V. mimicus*, *V. proteolyticus V. parahaemolyticus* and *V. cholera*. Cluster A was represented by 12 strains of *V. vulnificus* (Fig. 4).

Characterization of the resistance/susceptibility profile of the isolates was determined by disc diffusion method (Table 2). Out of 59 (100%) isolates 57.58% of the isolates were

Table 2: Antibiogram analysis of *Vibrio* strains from river Narmada.

Isolate No.	Name of species	Drug resistance profile
BGCC662	*V. cholerae*	Co, Ct, Na, E, Nx, Cu
BGCC663	*V. cholerae*	A, Cf, T, ct, Ak, E, C, Cu
BGCC664	*V. cholerae*	A, ct, Ak, E, Nx
BGCC665	*V. cholerae*	T, Ct, E, Cu, Do
BGCC666	*V. cholerae*	A, T, Ak, E, Cu
BGCC667	*V. cholerae*	Ct, E, C, Cu
BGCC668	*V. cholerae*	A, Ak, Na, E, Nx, Cu, Do
BGCC669	*V. cholerae*	A, Ak, E, C, Cu
BGCC670	*V. cholerae*	A, T, C, Cu, Nx, Cu
BGCC671	*V. cholerae*	A, Co, Tr, Na ,C, Cu, S
BGCC672	*V. cholerae*	A, Ct, E, C, S
BGCC673	*V. parahaemolyticus*	A, Co, Ct, Tr, Na
BGCC674	*V. parahaemolyticus*	T, E, C, Cu, Do
BGCC675	*V. parahaemolyticus*	A, T, Na, E, Cu
BGCC676	*V. parahaemolyticus*	Ak, E, C
BGCC677	*V. parahaemolyticus*	A, Ct, T, Ak, Na, E, Cu
BGCC678	*V. parahaemolyticus*	A, Ct, Ak, Tr
BGCC679	*V. parahaemolyticus*	A, Co, Ct, Tr, Cu
BGCC680	*V. parahaemolyticus*	A, T, Ak, E, Cu, S
BGCC681	*V. parahaemolyticus*	A, T, Ak, E, C, Cu, Do
BGCC682	*V. parahaemolyticus*	A, Ct, Na, E, C, Cu
BGCC683	*V. vulnificus*	Co, T, Na, C, Cu, Do
BGCC684	*V. vulnificus*	Cf, Ct, Tr, E, C, Do, S
BGCC685	*V. vulnificus*	A, Co, Cf, Ak, Tr, Na, C, Do
BGCC686	*V. vulnificus*	A, Co, Ct, Tr, Cu
BGCC687	*V. vulnificus*	Ct, Ak, E, Cu, Do
BGCC688	*V. vulnificus*	Co, Ct, Tr, C
BGCC689	*V. vulnificus*	A, Co, Tr, C, Do
BGCC690	*V. vulnificus*	Co, T, Ct, Na, E, C, Do, S
BGCC691	*V. vulnificus*	A, Cf, T, Ct, Ak, E, C, S
BGCC692	*V. vulnificus*	Co, Tr, Nx, Cu, Do,S
BGCC693	*V. vulnificus*	Co, T, Na, C, Cu, Do
BGCC694	*V. vulnificus*	Cf, Ct, Tr, E, C, Do, S
BGCC695	*V. fischeri*	A, Cf, Ct, Nx, Do
BGCC696	*V. fischeri*	Na, Cu
BGCC697	*V. fischeri*	E, C, Cu, Do
BGCC698	*V. fischeri*	Ct, Tr, E, C, Cu
BGCC699	*V. fischeri*	Cf, T, Na, E, C, Cu, Do
BGCC700	*V. fischeri*	A, T, Na, E, Nx, C
BGCC701	*V. fischeri*	Cf, Ct, Ak, Tr, Nx, C, Cu, S
BGCC702	*V. fischeri*	A, E, Nx, Cu, S
BGCC703	*V. fischeri*	A, Co, Tr, Nx, C, Cu, Do
BGCC704	*V. fischeri*	A, T, Ak, E, Nx, Cu, S
BGCC705	*V. fischeri*	E, Nx, Cu, S
BGCC706	*V. proteolyticus*	Co, T, Ct, Tr, E, Cu, S
BGCC707	*V. proteolyticus*	A, Tr, Cu
BGCC708	*V. proteolyticus*	A, Co, Ct, Tr, Na, E, C, Do, S
BGCC709	*V. proteolyticus*	Co, Ct, Tr, Na
BGCC710	*V. proteolyticus*	Cf, Ct, Ak, Na, E, Cu
BGCC711	*V. proteolyticus*	Co, T, Ct, Ak, E, C, Cu
BGCC712	*V. proteolyticus*	Co, Ak, Tr, Cu, Do, S
BGCC713	*V. proteolyticus*	A, Co, Cf, Tr, E, Nx, C, Do
BGCC714	*V. proteolyticus*	Ct, Na, E, Nx, C, Cu, S
BGCC715	*V. proteolyticus*	Co, Cf, Ct, Nx
BGCC716	*V. mimicus*	A, Co, Cf, T, Tr, Nx, Cu, Do, S
BGCC717	*V. mimicus*	Cu, Ct, Tr
BGCC718	*V. mimicus*	Ak, Tr, E, C, Cu, Do
BGCC719	*V. mimicus*	E, C, Cu, Do
BGCC720	*V. mimicus*	A, Co, T, Nx, Cu, Tr, Do, S

T: Tetracycline, A: Ampicillin, Ct: Co-trimoxazole, C: Chloramphenicol, Na: Nalidixic acid, Nx: Norfloxacin, Cf: Ciprofloxacin, Ak: Amikacin, Ca: Ceftazidime, Cu: Cefuroxime, Tr: Trimethoprim, S: Streptomycin, E: Erythromycin, Do: Doxycycline hydrochloride

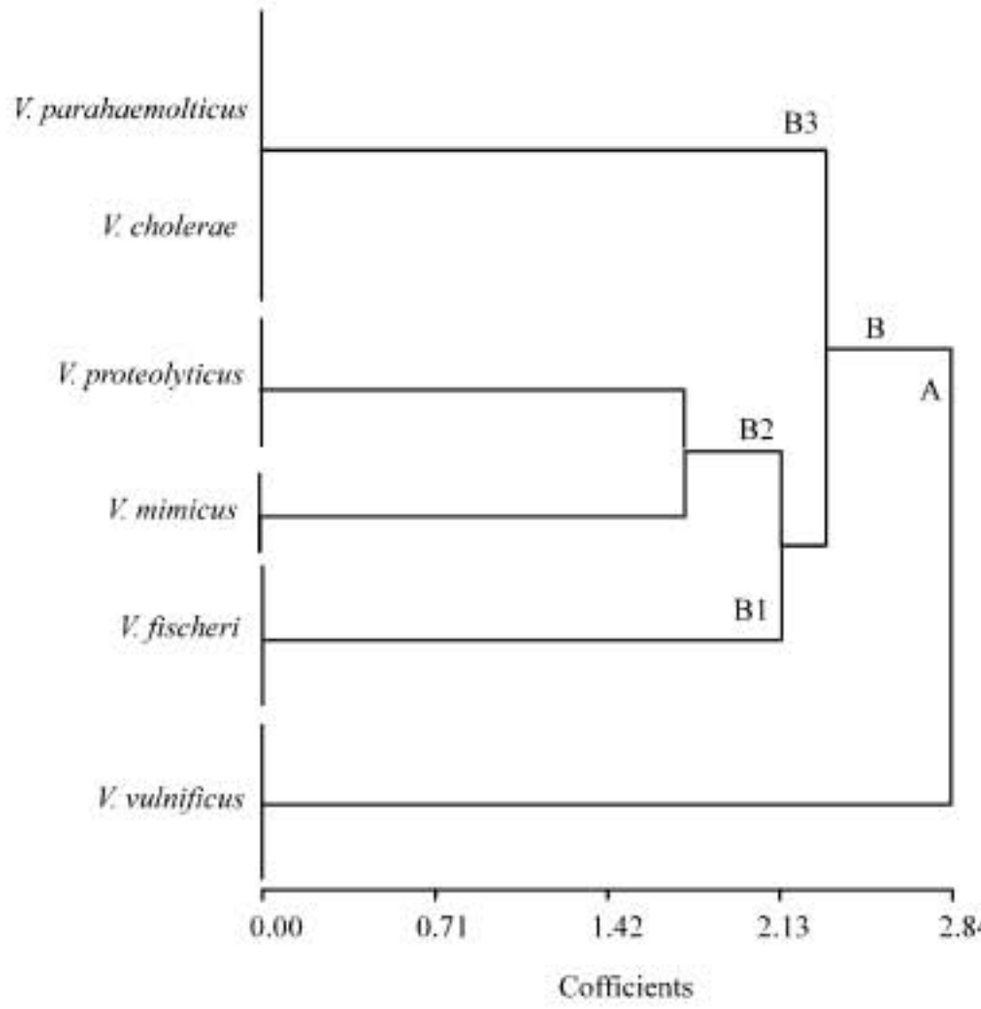

Fig. 4: UPGMA cluster analysis on the basis of RAPD of *Vibrio* isolates isolated from the River Narmada

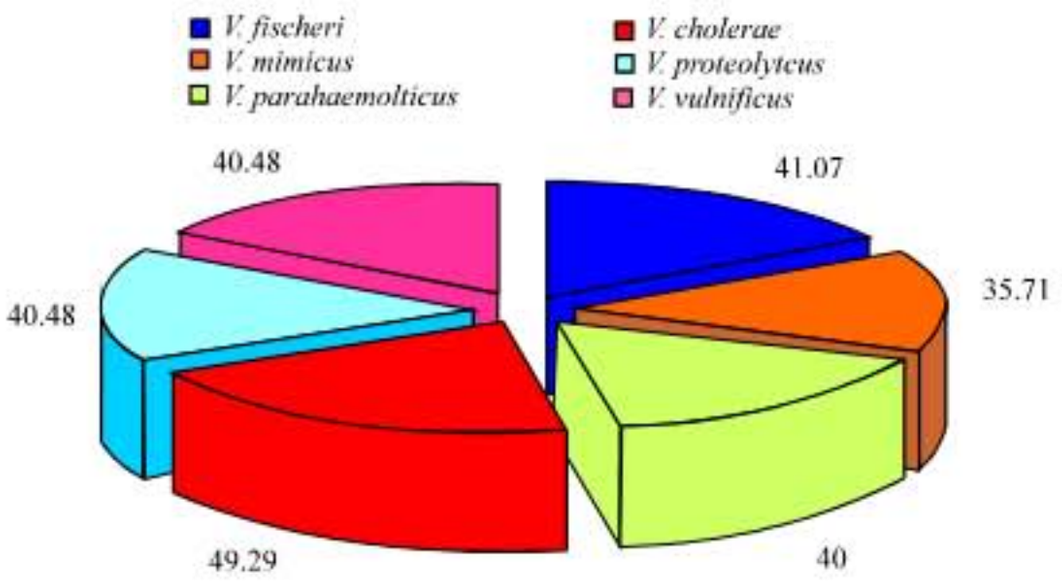

Fig. 5: Multiple drug resistance pattern exhibited by different *Vibrio* species isolated from River Narmada

resistant to Ampicillin, 33.33% to Cotrimoxazole, 48.48% to Amikacin, 54.56% to Ceftadizime, 84.85% to Erythromycin, 21.2% to Doxycycline hydrochloride, 18.18% to Streptomycin, 66.67% to Chloramphenicol, 78.79% to Cefuroxime, 33.33% to Nalidixic acid, 33.30% to Tetracycline, 66.67% to Ciprofloxacin, 9.09% to Norfloxacin to and 36.36% to Trimethoprim. The percentage multiple resistance exhibited by the isolates of *V. fischeri*, *V. mimicus*, *V. parahaemolyticus*. *V. cholerae*, *V. proteolyticus* and *V. vulnificus* were 41.07, 35.71, 40, 49.21, 40.48 and 40.48%, respectively (Fig. 5).

In the present study, out of 59 *Vibrio* isolates 6 kb plasmid was observed in only 11 *Vibrio* isolates belonging to single strain of *V. mimicus* (BGCC#718), *V. vulnificus*

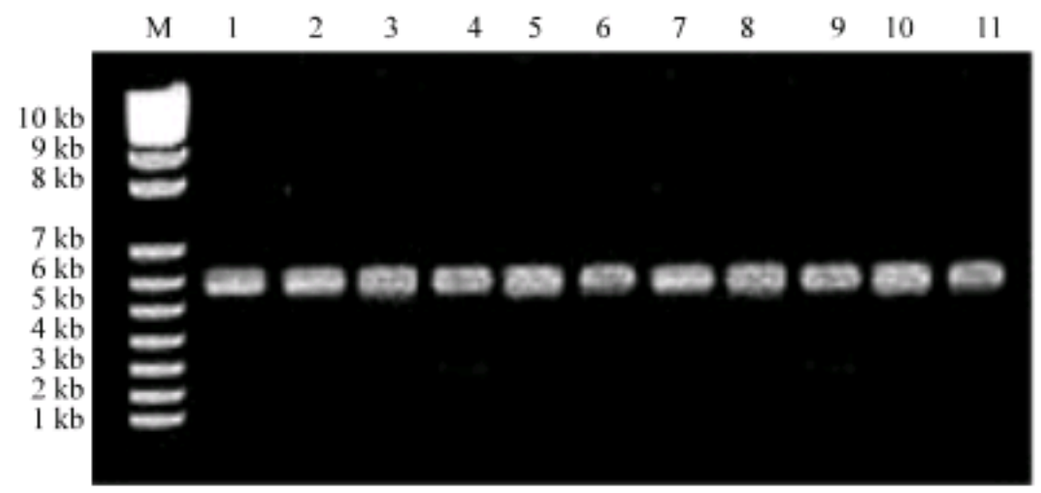

Fig. 6: Plasmid profiling of *Vibrio* isolates, M: Marker, Lane 1-11: BGCC# 718, BGCC# 710, BGCC# 718, BGCC# 690,BGCC# 693, BGCC# 690, BGCC# 703, BGCC# 662, BGCC# 664, BGCC# 670, BGCC# 671

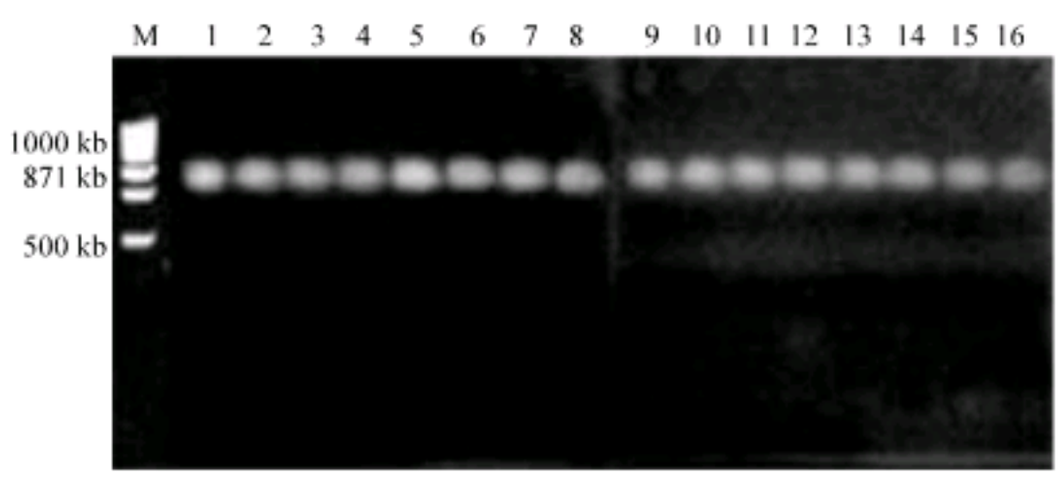

Fig. 7: Class 1 integron detected by PCR amplification, M: Marker, lane 1-16: BGCC# 785 BGCC# 695, BGCC# 697, BGCC# 706 ,BGCC# 715, BGCC# 707, BGCC# 700, BGCC# 663, BGCC# 668, BGCC# 669,BGCC# 672, BGCC# 673, BGCC# 675, BGCC# 676, BGCC# 678, BGCC# 682

(BGCC#690), *V. proteolyticus* (BGCC#710), two strains of both *V. parahaemolyticus* (BGCC# 679, BGCC# 680) and *V. fischeri* (BGCC# 690, BGCC# 703) and four strains of *V. cholerae* (BGCC# 662, BGCC# 664, BGCC# 670, BGCC# 671) (Fig. 6).

In the present study, class 1 integron (intI 1) was observed in single strain of *V. vulnificus* (BGCC#685), two strains of each of *V. fischeri* (BGCC# 695, BGCC# 697), *V. proteolyticus* (BGCC# 706, BGCC# 715), *V. mimicus* (BGCC# 717, BGCC# 720), four strains of *V. cholerae* (BGCC# 663, BGCC# 668, BGCC# 669, BGCC# 672) and five strains of *V. parahaemolyticus* (BGCC# 673, BGCC# 675, BGCC# 676, BGCC# 678, BGCC# 682) (Fig. 7).

SXT element is responsible for resistance to Co-trimoxazole, Trimethoprim and Streptomycin in *V. cholerae* (Waldor *et al*., 1996). However, inspite of antibiotic resistance against Co-trimoxazole, Trimethoprim and Streptomycin among the *Vibrio* isolates viz BGCC# 692, BGCC# 706, BGCC# 708, BGCC# 716 and BGCC# 720, the prevalence of SXT element were not found during the present investigation. Apparently this element made no contribution to resistance performance in these isolates.

DISCUSSION

In this study, ARDRA and RAPD were used as molecular tools to characterize the spatial phylogenetic diversity of the identified *Vibrio* strains. ARDRA has been used as an efficient

tool for molecular characterization and epidemiological study for a number of pathogens including *Salmonella enterica* (Olsen *et al*., 1992), *Staphylococcus aureus* (Blumberg *et al*., 1992), *Klebsiellia* (Sharma *et al*., 2007), *Enterobacter* sp., *Shigella* sp. (Sharma *et al*., 2009) *Vibrio* sp. (Unpublished data). Pourshafie *et al*. (2007) reported the presence of six different RAPD patterns among the 50 *Vibrio cholerae* O1 strains. Their data indicated a substantial discriminator power of PCR amplification of random fragments of genomic DNA using arbitrary primers of 10-15 mer. Unny *et al*. (2000) studied genetic diversity of *Vibrio cholerae* in Chesapeake Bay. Arias *et al*. (1998) created a RAPD profile database for *V. vulnificus* and compared with the ones obtained by ribotyping. They found a good correspondence between the two typing techniques. However, in the present study RAPD was unable to differentiate between *V. parahaemolyticus* and *V. cholera* as both were grouped in the same clade. Results may differ from previous study because primers which were used in present study not effective against clonal generations of *Vibrio* species. The ARDRA profile delineated the 6 *Vibrio* spp. into different clades, thus establishes the superiority of ARDRA over RAPD as tool for molecular characterization.

The emergence and spread of antibiotic resistance among human pathogens is certainly the most striking evolution that has arisen in bacteria. The result of plasmid analysis indicated that plasmid the 6 kb could be responsible for resistance in *Vibrio* sp. against β-lactum and broad spectrum cephalosporins. Zhang *et al*. (2006) isolated multidrug resistant environmental plasmid bearing *Vibrio* species isolated from polluted and pristine marine reserves of Hong Kong. They observed that all the plasmid bearing *Vibrio* sp. were resistant to twenty-one antibiotics tested against them. Later and Zhang *et al*.(2007) reported the presence of small plasmid (3.8 kb) in *Vibrio cholera* MP-1 isolated from marine environment. Cecerelli and Colombo (2007) demonstrated the importance of mobile genetic elements in acquisition of new genetic information and genetic recombination. The study involved the analysis of integron and Integrating Conjugative Elements (ICEs) of SXT/R391 family in relation to the transfer mechanism of mobile genetic elements.

The detection of class 1 integron indicates the presence of *dfrA1* and *aadA1* gene cassettes, which are responsible for the development of resistance against Trimethoprim and Aminoglycoside or both. Chunyan *et al*. (2008) reporterd incidence of diverse integrons and β lactamase genes in environmental enterobacteriaceae isolates from Jiaozhou Bay, China. Taviani *et al*. (2008) reported the presence of a polymorphic group of integrative conjugative elements and class 1 integron in environmental *Vibrio* sp. isolated from environmental samples in Mozambique. Jain *et al*. (2008) studied multidrug resistance in *V. cholerae* O1 strains associated with large outbreak in Orissa (Eastern India) and reported that class 1 integron and SXT element plays important role in emergence of multidrug resistant strains. Hochhut *et al*. (2001) also described a SXT element unable to confer a phenotypic pattern, because the resistance cluster was entirely deleted. Miyazoto *et al*. (2004) studies multidrug resistance *Vibrio cholerae* non O1 and non O139 from environment sources in Lao people's democratic republic. They did not find any mobile genetic elements reflecting antibiogram of the strains, SXT element is occasionally lacking the drug resistance gene, but they can acquire later. Therefore, SXT element should be monitored in *Vibrio* sp. from environmental sources. The study provides evidence of multidrug resistant strains present in *Vibrio* sp. of river Narmada. Furthermore mobile genetic elements viz. plasmid, class1 integron associated with drug resistance were found. However, none of the typical SXT resistance gene was reveled. Therefore role of mobile genetic elements in *Vibrio* genome plasticity should be reconsidered, particularly in relation to emergence of new pathogens along with the horizontal acquisition and dissemination of antibiotic resistant genes.

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