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Microbiota Diversity, Screening and Molecular Characterization of *Shigella* sp. from Estuarine Fishes

G. Jasmin Bilba Rani* • **S. Viveka**

Department of Biotechnology, Udaya School of Engineering, Vellamodi, Kanyakumari, Tamil Nadu, 629 204, India

Corresponding author: * **gbilbamsc05@yahoo.co.in**

ABSTRACT

An attempt has been made in the present study to assess the gut microbial diversity of fishes through biochemical and molecular approaches. Fishes such as *Mugil cephalus*, *Tilapia mosambicus*, *Etroplus suratensis*, *Mystus gulio* and *Cranx ignobilis* were collected from an estuarine environment. The influence of UV mutation on enzymatic activity was studied. Finally, the variation in molecular genetics of both wild and mutant *Shigella* sp. was assessed by RAPD-PCR analysis. Total viable colonies in the gastro-intestinal tract varied considerably. The average CFU/g gut sample was maximum (3.0 CFU/g) in *M. cephalus* and minimum (0.76 CFU/g) in *M. gulio*. Altogether 12 bacterial genera were identified and *Shigella* sp. was characterized for its extracellular enzyme production, indicating that production was dependent on host species. Further, a study of mutagenesis in this species indicated that non-producers of enzyme turned to producers. The RAPD-PCR profile of wild and mutant strains of *Shigella* sp. indicated the existence of 6 bands with a base-pair range of 233 to 1871. Thus, UV mutagen altered the enzymatic characteristics as well as the amplification of DNA fragments in *Shigella* sp.

Keywords: enzyme production, gastrointestinal tract, mutagenesis, RAPD-PCR

INTRODUCTION

Fishes dominate the tropic pyramid in most lakes, streams and estuaries. They occupy several different levels of the aquatic food chain and comprise over 40% of earth's vertebrate species (Feldhusen 2000). The microflora of a fish is a reflection of the aquatic environment in which it lives and colonization by pathogenic and non-pathogenic bacteria on the body surface, gills and intestine was reported by Railly and Kafertein (1997). Intestinal flora of endothermic fishes serves both as a digestive function and protection barrier against diseases (Sisson *et al*. 1989). This protection is likely to be mediated by microorganisms that are present in high numbers. The microbial relationship between environment and fish microflora is also a tool for monitoring change in fish forms (Allen *et al*. 1993). The gastrointestinal tract of a fish is a nutrient-rich habitat suitable for microbial growth and also the major route of fish infection. It does not favour microflora due to the action of defense mechanisms through the digestive enzyme lysozymes and immunoglobulins in gut mucous even though the gut microflora withstands all the above and establish in the gut environment was revealed by Cahill in 1990.

Protein secretion systems are essential virulence factors of many bacteria pathogenic to humans, animals and plants. These systems mediate the transfer of bacterial virulence proteins directly into the host cell cytoplasm (Akeda and Galan 2005). Many bacterial pathogens use the type III secretion system to inject "effector" proteins into host cells and 24 members of the effector protein family found in pathogens including *Salmonella*, *Shigella* and enteropathogenic *Escherichia coli* were reported (Alto *et al.* 2006).

Gram-negative bacteria commonly interact with eukaryotic host cells using type III secretion systems (TTSSs or secretons) which comprise cytoplasmic, transmembrane and extracellular domains (Cordes *et al.* 2005). These risks have prompted growing interest in probiotic strains that would hinder undesirable bacteria from colonizing the fish gut (Flint 1998). Most studies investigated the microfloral composition based on culturable bacteria. A characteristic of indigenous bacteria in many environments is the lack of culturability of the majority of living bacteria. PCR-based methods have been developed to rapidly screen bacteria as an alternative to time-consuming culture techniques (Delgado-Viscogliosi *et al.* 2009). Furthermore, the enzymes that are derived from these organisms can be potentially used in several industrial processes such as cloth washing (Dambmann and Aunstrup 1981) and molecular biology applications (Kobori *et al*. 1984). The extracellular protease of *Pseudomonas maltophila* was partially purified by ammonium sulfate precipitation and chromatography (Boethling 1975). The halophilic marine *Pseudomonas* sp. was purified by ammonium sulfate precipitation, DEAE cellulose, chromatography and gel filtration through Sephadex G-200. Norguist *et al.* (1990) isolated a mutant of the fish pathogenic bacterium *Vibrio anguillarum* that had a lower level of protease activity. Lipase and esterase have been recognized as biocatalysts because of their wide-ranging industrial applications, their stability and non-requirement for co-factors. A fast and simple method of lipolytic activity is based on the use of 4-methyl umbelliferone (Muf) derivative substrates, a useful tool for characterization of a variety of lipases from microbial origin (Nuriaprim *et al*. 2003). Random amplified polymorphic DNA (RAPD) is a DNA polymorphism assay based on the amplification of random DNA segments with single primers of arbitrary nucleotide sequence. RAPD has greater advantage since no prior knowledge of the target sequence is required for design of primers and the results are directly read from agarose gel (Williams *et al.* 1993). There are now many publications describing the development of molecular techniques for the examination and identification of fish and shellfish pathogens. These include diseases of bacterial, viral, parasitic and fungal origin. This review summarizes the published methods for molecular diagnosis of diseases listed under European Commission (EC) fish health regulations (Cunningham 2007). In the present work we selected five fish species and enumerated their intestinal microbes. Mutation

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Research Note

was induced and the enzymatic activity was screened to investigate the changes in RAPD profile between wild and mutant strains.

MATERIALS AND METHODS

Sample collection

Fish 15 to 20 cm in length were collected from Kadiyapattanum estuary of Kanyakumari District, Tamil Nadu. Five fishes were identified up to the species level by using standard keys described in the FAO fish identification catalogue (Jereb *et al.* 2001). The fish species chosen for the present study were *Mugil cephalus* (mullet), *Tilapia mossambicus* (tilapia), *Etroplus suratensis* (pearl spot), *Mystus gulio* (cat fish) and *Caranx ignobilis* (carangeides).

Measurement of fishes

The total length of fishes was measured by using a campus divider and measurement scale and expressed in cm. The body weight and gut weight of the chosen fishes were measured by using a top-loading electronic balance to an accuracy of 0.1 mg.

Enumeration of intestinal microbes

After collection, the fishes were transported to the laboratory under aseptic conditions and dissected. Then the gastrointestinal tract was immediately removed under sterile conditions and used for further analysis. After measuring the weight of the gastrointestinal tract, it was opened aseptically and grounded well using phosphate buffered saline (PBS) to a concentration of 5%. This was used as a stock solution for enumerating microbes. Then one ml of stock solution was serially diluted up to six dilutions (10^{-1} to 10^{-6}). From each dilution 1 ml of aliquot was seeded in nutrient agar by the pour plate method and incubated at 35°C in an incubator for 24 hrs. After enumeration, individual colonies of different colony morphology were isolated and identified based on biochemical tests (listed in **Table 4**; Cappuccino and Sherman 2005) and stored in a refrigerator (4°C) on nutrient agar slants until further use.

Screening of extracellular enzymatic activity

1. Natural selection

Five wild type strains of *Shigella* sp. were subjected to natural selection growing them on LB agar and incubating them for 24 hrs at 37°C.

2. UV irradiation

UV mutation was induced by exposing the 5 strains to UV light (3084LM) from 60 cm away for 25 min. These wild and mutant strains were screened for enzymatic activity.

3. DNA isolation

One ml of nutrient broth culture of different isolates of *Shigella* sp. was centrifuged at 10,000 rpm for 10 min. The pellet was resuspended in 1 ml of sterile distilled water and centrifuged at 10,000 rpm for 10 min. The pellet was collected and ground with 5 ml of CTAB (cetyl methyl ammonium bromide) DNA extraction buffer in a glass homogenizer. The mixture was emulsified with an equal volume of phenol: chloroform and centrifuged at 10,000 rpm for 5 min at room temperature. The aqueous phase collected was then added with an equal volume of cold absolute ethanol and the DNA was allowed to precipitate by keeping the tubes at -20°C overnight. DNA pellets were air dried. Pellets were dissolved in 5 ml of TE buffer. Then the isolated DNA was quantified by a spectrophotometer at 260 nm and quality was tested by agarose gel electrophoresis.

Table 1 Morphometric and gravimetric measurements of gastrointestinal tract of selected estuarine fishes.

Fishes	Total length of fish (cm)	Total weight of fish (g)	Total gut weight (g)
Mugill cephalus	18.5	125	3.38
Tilapia mossambicus	16.8	145	2.81
Etroplus suratensis	15.3	75	1.62
Mystus gulio	19.7	80	2.48
Caranx ignobilis	15	50	1.73

RAPD–PCR analysis

20 ng of DNA was dissolved in 20 µl PCR reaction buffer containing 10 mM Tris HCl (pH 9.0) 15 mM $MgCl_2$, 50 mM KCl and 0.01 g gelatin, 0.2 mM dNTPs, 21 pM of primer, 0.5 µg of DNA polymerase and 10 primers (RAPD kits A1 to A10) from Integrated DNA Technologies (IDT) were used for RAPD-PCR.

Amplification was performed using a programmable Thermal cycler pTc 150 (MJ Research, Watertown, USA) and the products resolved in 1.6% agarose gel, stained with ethidium bromide 1 µg/ml and run for 4 hr at 50V. The resulting photographs were captured by a gel documentation system (Herolab, Wiesloch, Germany). All fragments generated by RAPD-PCR using primers were considered and analyzed separately for determining the RAPD types. The size of each band was determined using Kodak Digital Science 1D (KSD1D 2.0, Rochester, USA) software and the presence (1) or absence (0) of a particular band was recorded to generate a binary table. The data table was exported into Bioprofile ID software (Sambrook *et al*. 1989) for analysis. The normalized RAPD pattern was further analysed using Gel compare II version 2.5 (Applied Maths, St. Martens-Latem, Belgium). The levels of similarity in the RAPD fingerprints were calculated using Pearson's correlation coefficient. Cluster analysis was performed using the unweighted pair group method with the arithmetic mean (UPGMA) method. The relationships between the pattern profiles were displayed as dendrograms and expressed as percentage similarity (Thomas *et al*. 2002).

Statistical analyses

Morphometric and gravimetric measurements of estuarine fishes, total viable count composition and distribution of aerobic heterotrophic population were expressed as the mean of three measurements.

RESULTS

Morphometric and gravimetric measurements of gastrointestinal tract of estuarine fishes were shown in **Table 1**. Gut microbial diversity study of chosen estuarine fishes such as *Mugil cephalus*, *Tilapia mossambicus*, *Etroplus suratensis*, *Mystus gulio* and *Caranx ignobilis* was reported in **Table 2**.

Table 2 Total viable count (CFU/ml) of aerobic heterotropic bacterial population in gastrointestinal tract of selected estuarine fishes.

Fishes	Dilutions						Average
	10^1	10^2	10^3	10^4	10^5	10^6	CFU/ml × 10^6
Mugill cephalus	TNTC	148	TLTC	28	12	8	3.00
Tilapia mossambicus	TNTC	TNTC	TLTC	16	9	5	2.50
Etroplus suratensis	TNTC	253	58	23	13	6	2.61
Mystus gulio	TNTC	TNTC	TLTC	18	12	9	0.76
Caranx ignobilis	TNTC	TNTC	42	19	10	7	2.74

TNTC : Too numerous to count
TLTC : Too little to count

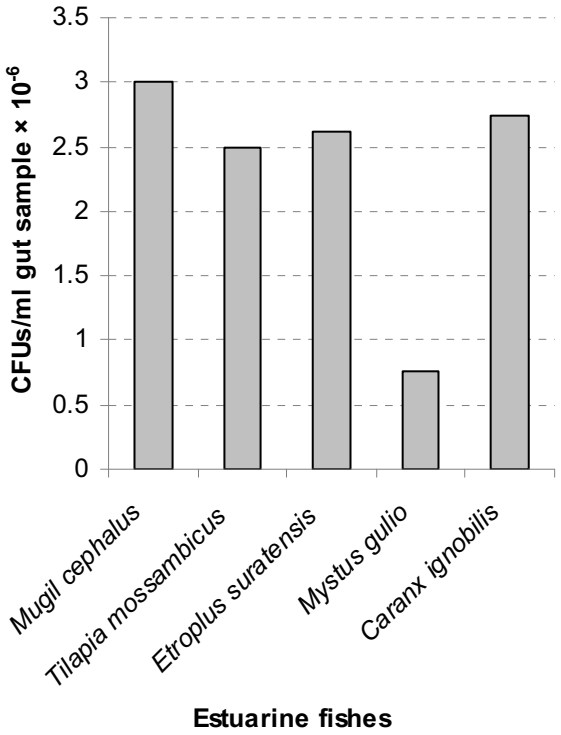

Fig. 1 Average colony forming units (CFUs/ml gut sample) of bacterial population isolated from estuarine fishes.

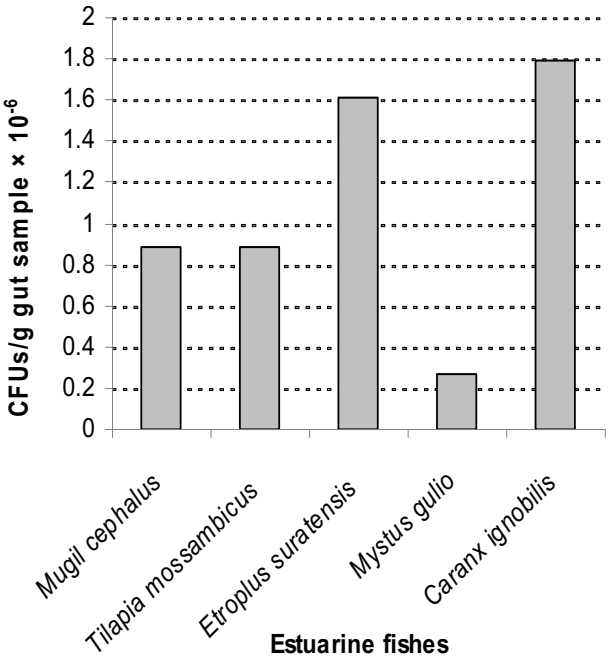

Fig. 2 Average colony forming units (CFUs/g gut) of bacterial population isolated from estuarine fishes.

The microbiota diversity of estuarine fishes *Mugil cephalus*, *Tilapia mossambicus*, *Etroplus suratensis*, *Mystus gulio* and *Caranx ignobilis* was 3×10^6 CFU/ml, 2.5×10^6 CFU /ml, 2.61×10^6 CFU/ml, 0.76×10^6 CFU/ml and 2.74×10^6 CFU/ml respectively (**Fig. 1**). The average numbers of bacterial colonies in the gut sample of respective fishes are 0.89×10^6 CFU/g, 0.89×10^6 CFU/g, 1.61×10^6 CFU/g, 0.27×10^6 CFU/g and 1.79×10^6 CFU/g (**Fig. 2**).

Strain isolation

A total of 13, 20, 22, 18 and 15 strains were isolated in the gut samples of *M. cephalus*, *T. mossambicus*, *E. surantenis*, *M. gulio* and *C. ignobilis*, respectively, among which *Shigella* sp. accounted for 1, 1, 2, 1 and 1 colonies respectively (**Table 3**). Biochemical tests conducted to confirm *Shigella* sp. are reported in **Table 4**. Further, UV mutation and screening for extracellular enzyme production was conducted. After that both wild (1 to 5) and mutated (6 to 10) *Shigella* sp. were screened for enzymatic assay. A clear zone around the streaking of skim milk agar plates shows the production of protease. A clear zone around the streaked starch agar plate shows amylase activity. The clear zone with oil droplet around tributyrin agar plate shows the production of lipase enzyme. Both strains showed varied enzymatic activity (**Table 5**).

Table 4 Biochemical characteristics of *Shigella* sp.

Biochemical tests	Results
Gram staining	-
Motility	-
Oxidase	-
Amylase	+
Lipase	-
Urease	-
Lactose	-
Indole	+
Methyl red	+
Voges proskauer	-
Citrate-Simmons	-
Gelatin hydrolysis	+
Catalase	-
Hydrogen sulphide	-

+ : Positive
- : Negative

The induction of UV mutation in the 5 strains of *Shigella* sp. from different estuarine fishes altered the extracellular enzyme activity. The mutant type (6) reverted from a non-producer to a producer of protease. Likewise the mutant type (7) reverted from a non-producer to a producer of amylase. In few cases, wild type 2 and 3 reverted from a pro-

Table 3 Distribution and composition of heterotropic bacterial genera in the gastrointestinal tract of selected estuarine fishes.

Bacterial genera	Fish species				
	Mugill cephalus (№ of colonies)	*Tilapia mossambicus* (№ of colonies)	*Etroplus suratensis* (№ of colonies)	*Mystus gulio* (№ of colonies)	*Caranx ignobilis* (№ of colonies)
Escherichia coli	3	1	2	2	3
Streptococcus	1	1	2	2	1
Staphylococcus	1	2	3	3	1
Micrococci sp.	-	1	-	-	1
Pseudomonas aeuroginosa	1	2	2	1	2
Salmonella sp.	1	1	2	1	1
Serratia sp.	1	-	1	2	2
Shigella sp.	1	1	2	1	1
Vibrio alginolyticus	1	9	7	5	1
V.parahaemolyticus	1	2	-	1	-
Corynebacterium	1	-	-	-	1
Proteus sp.	1	-	1	-	1

Table 5 Extracellular enzymatic activity of *Shigella* sp.

Fish species	Protease		Amylase		Lipase	
	Wild	Mutant	Wild	Mutant	Wild	Mutant
Mugill cephalus	-	+	+	-	-	-
Tilapia mossambicus	+	-	-	+	-	-
Etroplus suratensis	+	-	-	-	-	-
Mystus gulio	+	+	-	-	-	-
Caranx ignobilis	+	-	-	-	-	-

+ : Presence of enzyme
- : Absence of enzyme

Table 6 Primers used for RAPD-PCR analysis

Code	Sequence (5′-3′)
A_1	CAGGCCCTTC
A_2	TGCCGAGCTG
A_3	AGTCAGCCAC
A_4	AATCGGGCTG
A_5	AGGGGTCTTG
A_6	GGTCCCTGAC
A_7	GAAACGGGTG
A_8	GTGACGTAGG
A_9	GGGTAACGCC
A_{10}	GTGATCGCAG

ducer to a non-producer of protease. Wild type 1 and 4 reverted from a producer to a non-producer of amylase.

Molecular characterization

In this study, RAPD-PCR primers used for analysis are shown in **Table 6**. Primers amplified 2-8 fragments with amplicon size ranging from 564–4268 bp. Banding pattern of DNA fragments of 2-8 numbers (Lanes 1 to 5 in **Fig. 3**) in wild strains and 2-6 numbers (Lane 6 to 10 in **Fig. 3**) in mutant strains of *Shigella* sp. was recorded from fishes *M. gulio*, *C. ignobilis*, *M. cephalus*, *E. suratensis* and *T. mossambicus.* Cluster analysis of primers A1 to A10 generated RAPD profiles separated the strain isolates at an average similarity value of 74%. Primers yielded different patterns which clustered the strain isolates into 10 RAPD types. Dendrogram based on the combined similarity matrix generated with the RAPD primers is presented in **Fig. 4**. Data on similarity index (SI) based on RAPD profiles of both wild and mutant *Shigella* sp. indicated that the wild type *Shigella* sp. isolated from *E. suratensis* showed 0.57 SI with mutant of same and 0.75 SI with mutant strain from *T. mossambicus*. Mutant strains from *E. suratensis* and *M. gulio* showed the SI of 0.75. The wild strain from *C. ignobilis* showed SI of 0.80 with the mutant strain from the same fish (**Table 7**).

The result on dendrogram with homology co-efficient (%) showed that wild type (3) from *E. suratensis* has cluster with mutant (6) of *M. cephalus* (**Fig. 4**). Similarly wild type (4) from fish *M. gulio* form clusters with mutant strain (7) of *T. mossambicus* shows identical grouping but different profiles. Wild type (5) from *C. ignobilis* formed a single cluster with its own mutant strain (10) and indicated that mutation has no effect on this particular strain. The mutant strains (8, 9) from *E. suratensis* and *M. gulio* formed single

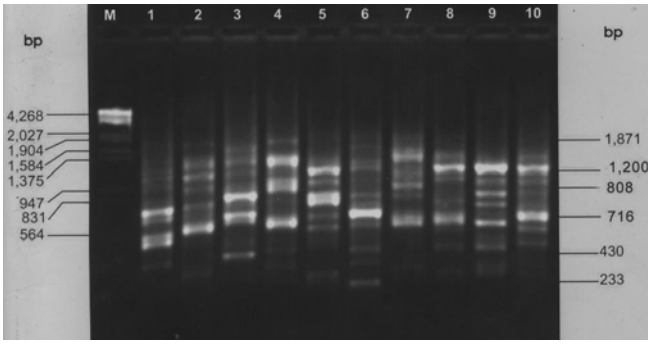

Fig. 3 Random Amplified Polymorphic DNAs of wild and mutant *Shigella* sp. generated by the primer RAPD Kit A10. M, Marker DNA; lanes 1-5: wild type *Shigella* sp.; lanes 6-10 mutant type *Shigella* sp.

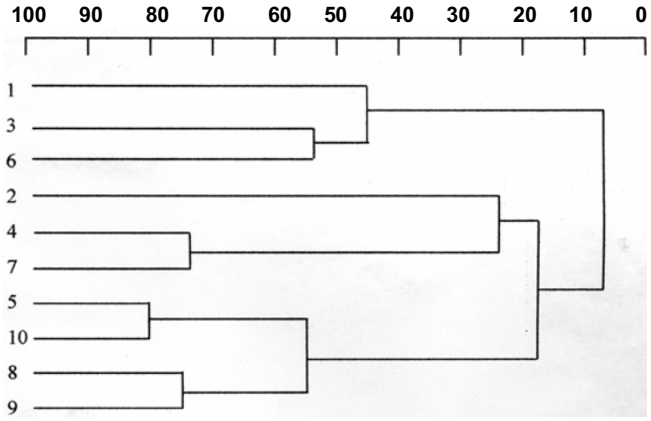

Fig. 4 Dendogram with homology co-efficient (UPGMA) of wild-type (1-5) and mutants (6-10).

cluster and represented as the same species.

Further wild type (1) of *M. cephalus* showed a solitary cluster with wild type (3) of *E. suratensis* and mutant type (8) of its own with 73% similarity. A similar trend was also noticed for wild type (2) of *T. mossambicus* with wild type (4) of *M. gulio* and mutant (8) of the same. Similarly wild and mutant strains (5, 10), (3, 8), (4, 9) of *C. ignobilis*, *E. suratensis* and *M. gulio* separately formed a solitary cluster.

Table 7 Similarity based on RAPD profiles of wild (1-5) and mutant (6-10) *Shigella* sp. of estuarine fishes.

	1	2	3	4	5	6	7	8	9	10
1	0.00									
2	0.08	0.00								
3	0.40	0.22	0.00							
4	0.08	0.22	0.25	0.00						
5	0.33	0.20	0.22	0.22	0.00					
6	0.50	0.25	0.57	0.08	0.50	0.00				
7	0.08	0.22	0.50	0.75	0.15	0.29	0.00			
8	0.08	0.15	0.55	0.29	0.50	0.33	0.29	0.00		
9	0.08	0.20	0.22	0.22	0.60	0.50	0.67	0.75	0.00	
10	0.08	0.20	0.44	0.08	0.80	0.75	0.67	0.75	0.60	0.00

DISCUSSION

It is well known that the digestive tracts of the aquatic organisms are colonized by a great number of bacterial floras. In the present study the total microbial diversity was analyzed in *in vitro* condition and the total viable count of selected fishes ranged from 1.22×10^4 to 1.97×10^6 CFU/ml. Bacteria able to survive and colonize the digestive tract are quite successful in maintaining large stable populations with typical numbers of aerobic bacteria in the range of 10^8/g of intestinal tissue and anaerobic bacteria approximately 10^5/g (Ringo *et al*. 1995). Among the bacterial colonies screened, twelve bacterial genera identified were *E. coli*, *Streptococcus*, *Staphylococcus*, *P. aeuroginosa*, *Salmonella* sp., *Shigella* sp., *Serratia* sp., *V. alginolyticus*, *V. harveyi*, *V. parahaemolyticus*, *Corynebacterium* and *Proteus* sp. The microbial populations within the digestive tract of fish are rather large in size. But they are considerably simpler in diversity than those found in endoderm (Ringo *et al.* 1995). The predominant species of bacteria in the intestine of marine fishes are *Aeromonas*, *Enterobacter*, *Flavobacterium*, *Pseudomonas*, *Achromobacter*, *Corynebacteria* and *Micrococus* (Cahil *et al.* 1990). The ecological studies on the intestinal bacteria have been undertaken by several authors in several fish species. The bacterial flora in fry and fingerlings of India's major carp Rohu (*Labeo rohita*) was reported by Das and Mukerjee (1999). The intestinal group of *Vibrio* sp. including luminous bacteria predominated during all seasons in the intestinal environment (Backert and Meyer 2006). These studies clearly indicated that the variations registered for TVC and bacterial composition in the selected estuarine fishes may be attributed to the eco-biological conditions prevailed in the intestinal environment of the respective fishes. The pathogens manipulate host cell functions to trigger bacterial uptake, facilitate intracellular growth and suppress defense mechanisms, thus facilitating bacterial colonization and disease development.

The present study on UV mutation provides noteworthy information about the extracellular enzyme production patterns. It is evident that mutation by UV radiation altered the extracellular enzyme production of the *Shigella* sp. isolated from the estuarine fishes. Mutagenic pollution of the environment is a serious and general problem. The variation of the physiological characters of an organism due to pre-existing factor in the microorganism or the changes in the extracellular enzyme production is due to acquired factors. In our study, the variation in the extracellular enzymes lipase, amylase and protease activities in the strains of estuarine fishes due to UV mutation induced changes. It concerns both the humans and the natural environment contaminated with mutagenic pollutions appearing mostly as a result of industrial processes. Mutagenic chemical occurring in various habits can induce serious diseases including cancer (Goldman and Stields 2003). The induction of mutation comparison of nadA and nadB in 14 *Shigella* strains and enteroinvasive *Escherichia coli* (EIEC) versus *E. coli* showed that at least one locus is altered in all strains (Prunier *et al.* 2007).

The assessment of diversity through molecular study RAPD Profile has been widely used for the genetic difference of plant and animal pathogen (Lipp and Alvarez 1992). RAPD-PCR was exploited using primers which gave reproducible and well distinguishable amplicons. The RAPD profiles obtained with primers individually, showed diversity among *Shigella* isolates used in this study. Cluster analysis based on combined similarity matrix grouped all strains into 9 clusters, indicating a genetically heterogeneous group of *Shigella* sp. to be prevalent along the estuarine. Our observations are in agreement with previous studies in reporting the presence of a large number of heterogenic genotypes within the sequences of four chromosomal genes (*trp*A, *trp*B, *pab*B and *put*P) and three virulence plasmid genes (*ipa*B, *ipa*D and *ics*A) in a collection of 51 *Shigella* and EIEC strains. The phylogenetic tree derived from chromosomal genes showed a typical "star" phylogeny indicating a fast diversification of *Shigella* and EIEC groups. Phylogenetic groups obtained from the chromosomal and plasmidic genes were similar suggesting that the virulence plasmid and the chromosome share similar evolutionary histories (Escobar-Paramo *et al.* 2003).

Molecular identification of microbes has been frequently conducted on the basis of demonstrative polymorphic DNA fragments amplified by PCR (Venieri *et al.* 2005). It has been suggested that the outcome of RAPD reactions was important and determined by a competitions for priming size in the genome (Williams *et al.* 1990). RAPD typing method allows the tracing of particular strains and its prevalence in different hosts (Maiti *et al.* 2009). The ability of RAPD-PCR to cluster *Shigella* strains suggested that this technique could be used in differentiating *Shigella* strains. The dendogram result confirmed the variation occurred in the DNA base pairs of the wild and mutant species. All the strains were grouped into 9 clusters. From this study, it was observed that among the generated amplicons, one amplicon of size 1200 bp was shared by 4 *Shigella* isolates studied. From the results, it is evident that the variation in host species also exerts changes in molecular genetics of strains. Yang *et al.* (2007) reported that phylogenetic tree was done using 23 house keeping genes from 46 strains of *Shigella* and enteroinvasive *E. coli* forms *Shigella* into 3 main clusters and 5 outliers , this strongly suggest that *Shigella* has multiple origin with *E. coli*. Their result findings showed that *Shigella* sp. with diverse genome but similar pathogenic properties.

Further the analysis of RAPD profiles resulted in a higher similarity value of 0.80. Hence we suggested the similarity relationship among the genotype of *Shigella* isolates. Maiti *et al.* (2009) reported that highly conserved fragments could be ideal for identifying strains that are atypical or which may be difficult to identify by phenotypic tests. Thus, it can be concluded that RAPD-PCR and dendrogram analysis are a rapid and simple tool could be used in genetic variation among the *Shigella* strains.

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