Original Research Article

Random amplified polymorphic DNA-PCR analysis on bacterial strains from children diarrhea

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A B S T R A C T

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activity.

This study Acute diarrhea in children is a leading cause of mortality in the developing countries. Molecular typing methods, due to their higher discriminating ability, may help in the better characterization of the Enteropathogenic *E.coli* and other isolates and these have been used worldwide. This study was aimed at subjecting the bacterial isolates from the stool samples to molecular typing methods like the Random Amplification of Polymorphic DNA (RAPD) and polymerase chain reaction (PCR). Bacterial strains (n=38), which were isolated over a period of six months from diarrheagenic stool samples. The bacterial strains were subjected to molecular typing methods like RAPD and PCR. Among the bacterial genera isolated, *E.coli* was recorded as the prominent, followed by the species of *Vibrio, Salmonella, Pseudomonas and Staphylococcus.* Of these diversified bacterial pathogens, *E.coli* was found one of the commonest causative agents of diarrhea. The higher percentage of incidence of *E.coli* along with the diarrhoeagenic organisms showed the significant health threat to the children in the study area.

Introduction

Global, regional and national estimates clearly place diarrhoeal diseases as a major, albeit a substantially neglected, public health problem worldwide as it is responsible for approximately 4 billion cases of diarrhea per annum, of which 2 billion cases result in death (Boschi *et al.,* 2008). Each year, more than 2 million children don't live to see their 5th birthday because of diarrhea and pneumonia

region, almost 48% of the estimated 3.07 million deaths annually are attributed to acute respiratory infections and diarrhoeal diseases with the highest burden of diarrhoeal disease in 5 countries: Bangladesh, India, Indonesia, Myanmar and Nepal where these diseases cause 60,000 deaths annually (Ghimire, 2008; Deen *et al.* 2008). Diarrhoea is a syndrome

that can be caused by different bacterial, viral and parasitic pathogens. The causative agents of diarrhea range from chemical irritation of the intestinal tract to infectious pathogens (Curtis *et al.,* 2000). The transmission of infectious is generally via the faecal-oral route (Nimri and Meqdam, 2004; Ghosh *et al.,* 1991). Among them bacteria represent approximately 61% having ability to cause diarrhea. The major cause of death for children is affected by diarrhoeagenic bacteria *E. coli, Vibrio, Salmonella* and *Shigella* are most common. Among the bacterial pathogens various strains of *E. coli* are the major culprits. *E. coli* is the most important etiological agent of childhood diarrhea and represents a major public health problem in developing countries (Nataro and Kaper, 1998). Other bacteria which are reported to cause gastroenteristis include *Salmonella* spp.*, Shigella* spp.*, Campylobacter* spp.*, Pseudomonas* spp.*, S. aureus* and *Vibrio* spp.

Epidemics of cholera have been reported from various part of India (Taneja *et al*., 2003). It is caused by *Vibrio cholera* and other species belonging to the genous *Vibrio* are well known as causative agent of gastrointestinal, extraintestinal wound infections with a fatal outcome in some cases (Dumontet *et al.,*, 2006). *Vibrio parahaemolyticus* is one of the most important food-borne pathogens in coastal countries. It is a halophilic Gram negative bacterium usually associated with seafood (Hin-Chung *et al.,* 2001). A high incidence of this pathogen undoubtedly originates from the frequent consumption of Marine foods in these countries. Clinical manifestations have included diarrhoea, abdominal cramps, nausea, vomiting, headache, fever, and chills, with incubation periods ranging from 4 to 9 hrs.

Staphylococcal aureus food poisoning is an illness that results from eating food contaminated with a toxin produced by the *Staphylococcus aureus* bacteria (Mead *et al.,* 1999). It exists in air, dust, sewage, water, milk, and food or on food equipment, environmental surfaces, humans, and animals. Designated to the Gram -negative group of bacteria, *Salmonella species* and *Pseudomonas aeruginosa* are frequently connected with food contamination. Apart from their association with diseases such as haemorrhagic colitis syndrome in humans (*E. coli*), foodborne toxic salmonellosis (*Salmonella* spp.,) and infections like folliculitis and infantile diarrhea (*P.aeruginosa*), they also share a characteristic membrane composition (Mary *et al.,* 2012; Whiteman and Bickford, 2007). Most types of salmonella live in the intestinal tracts of animals and birds and are transmitted to humans by contaminated foods of animal origin. A person can become infected with *Pseudomonas aeruginosa* numerous ways-through direct contact or indirect contact from contaminated surfaces. Notably, *P. aeruginosa* can survive from six hours to 16 months on dry, inanimate surfaces in hospitals. This study was aimed at subjecting the bacterial isolates from the stool samples to molecular typing methods like the Random Amplification of Polymorphic DNA (RAPD) and polymerase chain reaction (PCR).

Materials and Methods

Samples

Diarrhoeal stool samples were collected from children suffering from acute diarrhoea and gastroenteritis infection. The study was carried out for six months between July 2012-December 2012. The

samples were collected from the hospital and village primary health centers at Pattukkottai area. The stool specimens suspected of having bacteria as the etiological agent for diarrhoea alone were processed in the present investigation.

Isolation and identification

The diarrhoeal stool specimens were collected using sterile swabs with gentle scrap and put into the Stuart's transport medium (3 mL) in a submerged condition with ice box. A portion of stool sample was aseptically transferred to a sterile selective enrichment broth to study the incidence of bacterial enteric pathogens present in the diarrhoeal stool samples. The inoculum on the plate was streaked out for discrete colonies with a sterile wire loop.

The culture plates were incubated at 37°C for 24 hours and observed for growth through the formation of colonies. All the bacteria were isolated and identified using morphological, microscopy and the biochemical test was performed by Oxidase, Catalase reaction, Nitrate reduction, fermentation of sugars, Sucrose, Urease, Manitol, Haemolytic activity test, Thioglycollate citrate blue salts (TCBS), Eosine methylene blue agar (EMB), Brilliant green agar (BGA), *Salmonella Shigella* agar (SS), Cetrimide agar, Nutrient agar, Mannitol salt agar (MSA) medium were used for the selective isolation of *Vibro spp.*, *E. coli*, *Salmonella* spp., *Pseudomonas* spp., and *Staphylococcus* spp. (faecal) following standard procedures described by Sharma (2008).

Molecular typing by RAPD and ERIC PCR

Isolation of genomic DNA

Genomic DNA was isolated following the method. For isolation of DNA, overnight broth culture was prepared by inoculating the selected six (from each species) cultures into Luria Betanie broth. Gel electrophoresis was performed to confirm the presence of DNA. Agarose gel electrophoresis was the method used to separate macromolecules based on charge size and shape. The concentration was adjusted to 5ng/ml for PCR analysis

PCR and RAPD markers

Each PCR amplification was performed in a final volume of 20μl of reaction mixture containing, Template DNA 1 μl, Taq polymerase buffer 2 μl (Bangalore Genei, India), Taq DNA polymerase 1 μl, dNTP mix 2 μl and forward primer 1 μl 5-TCG GGA GGT G-3, Concentration amount of oligo nucleotide (5.2=56.80=0.17 OD 260 nmoles mg), Reverse primer 1 μl 5-TGC TCT GCC C-3, Concentration amount of oligo nucleotide (7.4=93.60=0.28 OD 260 nmoles mg), Reverse primer 1 μl 5-TGC TCT GCC C-3, Sterile water 12 μl. Amplifications were performed on a DNA with the cycling program consisting of step 1: initial denaturation at 95°C for 5 min, step 2: naturation at 94°C for 30 sec, step 3: Annealing 55°C for 1 min, step 4: extension 72°C for 1 min, step 5: go to step 2 for 35 cycles and finally at 72°C for 10 min extension. The amplified products were kept at 4°C until loaded on to the gel.

Amplification products were separated by electrophoresis on 1% agarose gel in 1XTBE buffer (pH 8.0) to which ethidium bromide (7 μl) was added in dissolved agarose.

Results and Discussion

Bacterial strains (n=38) which were isolated over a period of six months from diarrhoeagenic stool samples and it were received for routine culture sensitivity testing at the Gangasaras clinical laboratory, Pattukkottai, were included in the study. In the present investigation we have isolated selected strains of diarrhoeagenic bacterial species, which might have responsible for causing gasteroenteritis. All the samples were showed good growth on respective selective media. Based on their presumptive isolation of diarrhoeagenic bacterial strains were further subjected to biochemical tests for the identification of their genus (Table 3).

Among the 38 samples, significant number bacterial isolates have been isolated from stool samples. It was observed that 55.2 % of incidence of *E. coli* was recorded; it was the higher level of incidence while compared with others (Table 1). Whereas 47.3 % of incidence of *V. cholerae* were noticed on TCBS agar medium. It was also found that 44.7, 39.4, 31.5 % and 7.9% of incidence of *P. aeruginosa, S. typhi, V. parahaemolyticus* and *S. aureus* have been noticed respectively. The percentage incidence was highly significant among the children.

Haemolytic activity

In the present investigation, all the isolates of diarrhoeagenic strains from children diarrhoeal stool samples were subjected to haemolysin production by means of blood agar plate (Table 2). All the strains were varying in their ability of the different haemolysins (α and β) productions. It was observed that 17 strains of *E. coli* showed β haemolysin, and other strains were of significantly higher in the production of haemolysis.

For the analysis of RAPD profiles, we have selected the bacteria strains from each genera. All the strains were randomly selected and it β − haemolysin producers. The RAPD profile of the above mentioned strains are represented in plate 1. Bands were scored as present or absent. All the strains have produced bands with different molecular weights and it ranged from 0.5 to 1.3 kbp and the molecular weight was determined by comparing with 3000 bp DNA ladder (BangaloreGenei). The number of bands varied within the isolates, and it ranged between 2 and 6 bands. Even among the diarrhoeal isolates, no similar profiles have been observed, but all the bands were very clear. This revealed the wide genetic diversity of the diarrhoeagenic strains tested.

The template DNA of *E.coli* strains were randomly amplified and produced highly variable bands by the PCR and it showing four variable bands with the molecular weight 1300, 1050, 750 and 530 bp. In lane2 exhibited the same four bands they were identical from Lane1 and it comprises the molecular weight of about 2000, 1600, 1000 and 750 bp and the fourth band is exhibiting the coincidence of third band of the *E.coli*.

Vibrio cholerae and *V. parahaemolyticus* of amplified product showing the clear profile with the identical band of molecular weight 1800, 750, 530, 400 bp.

In lane 5 *S. aureus* showing the DNA strains of four bands with comprising the molecular weight 1100, 850, 720 and 420 bp and similarly in the Lane 6, there was three bands (1000, 800 and 416 bp) produced by the strain *P. aeruginosa*. In *V.*

Table.1 Percentage incidence of dirrhoagenic selected bacterial strains

Types of strains	Positive	Percentage
E. coli	21	55.2
S. aureus	3	7.9
S. typhi	15	39.4
V. cholerae	18	47.3
V.parahaemolyticus	12	31.5
P. aeruginosa	17	44.7

Table.2 Haemolytic activity

Strains	No	α	β	γ
E. coli	21	3	17	1
S. aureus	3	0	3	0
S.typhi	15	3	11	1
V.cholerae	18	9	3	6
V.Parahaemolyticus	12	6	4	2
P.aeruginosa	17	5	8	4

Plate .1.RAPD finger printing analysis. Lane M- Standard DNA ladder (1000-100bp) Lane1-*E.coli*, Lane-2 *staphi.aureus,* Lane-3 *Vibrio cholerae*, Lane-4 *Vibrio parahaemolyticus,* Lane-5 *Staphy.aureus*, and Lane-6 *Pseudomonas aeruginosa*

RAPD fingerprinting analysis of bacterial isolates of children diarrhoea
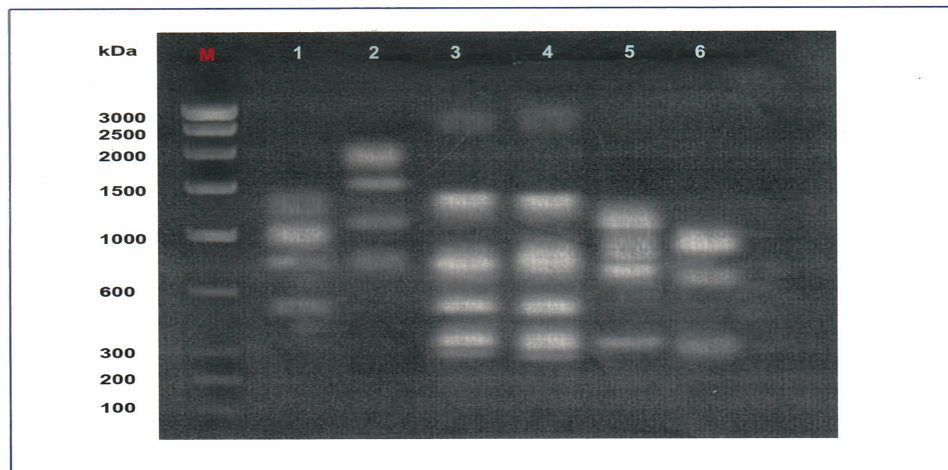
Table.3 Distinguishing biochemical reactions entero-pathogenic bacteria

Tests	*E.coli*	*S. aureus*	*S.typhi*	*V.cholerae*	*V.parahaemolyticus*	*P.aeruginosa*
Gram Staining	-	+	-	-	-	-
Motility Test	+	-	+	+	+	+
Indole production	+	-	-	+	+	-
Methylred	+	+	+	-	-	-
Voges-prospkauer	-	+	-	+	+	-
Citiate utilization	-	-	+	+	+	+
Oxidase	-	-	-	+	+	+
Catalase	-	+	+	-	-	+
Urease	-	+		-	-	+
Glucose	+	+		+	+	+
Manitol	+	+		+	+	-
Sucrose	+	+		-	-	-

cholarae and *V. parahaemoliticus* the second band showing coincidence of third band of *E.coli* and Lane 5 of fourth band shows coincidence of fourth band of *V. cholerae* and *V. parahaemoliticus*. These identical bands reveal the homogeneity between the strains.

In developing countries, pathogens have been identified in 65% of stool samples from children with acute diarrhea. In the present study, the percentage of diarrhoeagenic organisms isolated from the stool samples are *E. coli*, species of *Pseudomonas, Salmonella, Staphylococcus* and *Vibrio* spp. In the previous study the following group of bacteria viz. *Salmonella, Shigella, Aeromonas, Vibrio, Proteus, Enterobacter, Klebsiella and Pseudomonas spp.,* have been reported to cause diarrhea (Bravo *et al.,* 2003; Banto *et al.,* 2007). Among all the strains, the incidence level of *E. coli* was recorded as 55.2%. It was higher incidence when compared with others. In many instances the isolation of *E.coli* from stool samples of asymptomatic individuals was similar to that of the diarrhoeal cases (Carvalho, 2007; Dhanashree *et al.,* 2013). However, its association with several outbreaks and in volunteer studies has unquestionably confirmed the role of *E.coli* as an important aetiologic agent leading to diarrohea (Clarke *et al.,* 2003)

In the present investigations also the incidence of *Staphylococcus* spp. was recorded as considerably lower with 7.9%. Similar study conducted by Subashkumar *et al*., (2007), 5.43% incidence was recorded from children diarrhoeal samples. Another predominant bacterial genera was *Vibrio* spp, which has been shown as a major cause of epidemic diarrhoea. A significant level of incidence of *Vibrio* spp. was recorded in all the sampling areas

(47.3% of *V. cholerae*). This may be due to the tendency of this organism to cause severe diarrhea, thus making infected individuals more likely to seek medicinal attention ((Fasano, 2000)). In a recent outbreak of cholera in Chandigarh, India, it was recorded that 58.5% of children have been affected with acute diarrhea (Taneja *et al*., 2003).

RAPD-PCR profiles of diarrhoeagenic bacterial strains.

RAPD, one of the DNA fingerprinting methods, can be used in studies of epidemiology of foodborne diseases (Hill and Jinneman, 2000). In RAPD, a number of regions will be amplified that are bounded by nucleotide sequences showing similarity to the primers. RAPD is largely used for genetic variability analyses although on the other hand, there are some limitations (Bando *et al.,* 2007). Polymerase chain reaction (PCR) based techniques have been applied successfully to the epidemiological typing of various bacterial species. It is one of the molecular technique, which relies on the in vitro amplification of a DNA fragment and the result is obtained with in a short period of time after receiving the samples. Random amplification of polymorphic DNA (RAPD) is based on the amplification of random DNA segments with a single primer of arbitrary nucleotide sequence. Because of the low stringency inherent in this procedure, the patterns generated by RAPD may be affected by experimental parameters and standardization is crucial. RAPD analysis was chosen because it is reliable and can be use to differentiate between strain within the species.

A greater heterogeneity among diarrhoeagenic the strain has been

demonstrated by RAPD-PCR. Even though, all the isolates are of clinical origin, it was observed that none of the isolates produced identical profiles. This clearly reflects the genetic diversity of the strains tested. However, strains of *Vibrio* showed clear profile with similar type of banding patterns reveals the clear homogeneity among the strains.

Among the bacterial genera isolated, *E.coli* was recorded as the prominent, followed by the species of *Vibrio, Salmonella, Pseudomonas and Staphylococcus*. Of these diversified bacterial pathogens, *E.coli* was found one of the commonest causative agents of diarrhea. The higher percentage of incidence of *E.coli* along with the diarrhoeagenic organisms showed the significant health threat to the children in the study area. Haemolysin was considered as the major criteria for the selection of the strains for the molecular typing study and it was a random selection.

This study provides information about the occurrence and prevalence of bacteria associated with diarrhoeal episodes and its significant incidence was recorded among children. To the best of our knowledge, molecular typing has not been used in this most of the clinical laboratories in our country to discriminate bacterial isolates from stool samples. However, an attempt was made in the present study, the genetic similarities and the differences among the various groups of isolates from stool samples. Further studies which involve more number of samples and better discriminating molecular techniques are necessary to know the genetic similarities and the differences among the different diarrheagenic isolates.

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