Phenotypic and genotypic characterization of *Shigella* spp. with reference to its virulence genes and antibiogram analysis from river Narmada

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Abstract Water samples of the river Narmada from the source to the mouth were analyzed for the presence of shigellae and the *Shigella* isolates from 180 water samples were characterized by biotyping, serotyping and molecular typing. Out of all the 40 isolates, 23 were identified as *S. flexneri*, 10 as *S. sonnei* and 7 as *S. dysenteriae*. Serotyping was found to be the better identification method than biotyping. In the present investigation, amplified ribosomal DNA restriction analysis (ARDRA) with a probe complementary to 16S rRNA was performed. Repeated ARDRA analysis established the similarities between the isolates and thus suggested ARDRA as authentic and precise detection protocol. The isolates were also analyzed for the presence of virulence genes including *ipaBCD*, *ipaH* and *stx1*. All the 40 isolates of *Shigella* were found to be positive for the *ipaH* gene. The plasmid encoded invasion-associated genes *ipaBCD* were present only in *S. flexneri* and the *stx1* gene was found only in *S. dysenteriae*. This study demonstrated the existence of *Shigella* in the river Narmada and the dispersion of different virulence genes among the isolates, which appear to constitute an environmental reservoir of *Shigella*-specific virulence genes.

Keywords River water · *Shigella* · Serotyping · Molecular typing · Virulence gene

Introduction

Bacillary dysentery caused by *Shigella* is endemic throughout the world and it is among the most common causes of bacterial diarrheal diseases. It is responsible for approximately 165 million cases annually, of which 163 million are reported from developing countries and 1.5 million reported from industrialized ones. It is estimated that 1.1 million people die annually from *Shigella* infection and nearly 5,80,000 cases of shigellosis are reported among travelers from industrialized countries. The frequency of occurrence of *S. flexneri*, *S. sonnei*, *S. boydii* and *S. dysenteriae* were 60%, 15%, 6% and 6% in developing countries; and 16%, 77%, 2% and 1% in developed ones, respectively. In developing countries, the predominant serotype of *S. flexneri* is 2a, followed by 1b, 3a, 4a and 6 [1, 2]. *Shigella* contaminated food and drinks are often source of epidemic spread but very little is known about its presence and possible spread through environmental water. In many developing countries with inadequate sanitation, fecal contaminations of environmental water by enteric pathogens are very common. It is therefore important to understand whether *Shigella* can survive and persist in environmental water in the absence of a primate host and investigate the virulence characteristics of such environmental strains.

Identification of *Shigella* in environmental samples, where the number of organisms is likely to be small, is limited mainly by the lack of a suitable enrichment technique. Although DNA probes or PCR assay directed against the large invasion plasmid (*ipaBCD* and *ipaH*) or genes encoding shiga toxin (*stx1* and *stx2*) can be used to detect the presence of the organism but the isolation of live bacteria is essential to characterize their pathogenic potential

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as well as their sensitivity to antimicrobial agents [3–6]. Detailed analysis of a large number of water samples for the presence of *Shigella* by conventional culture methods is impractical, particularly because the number of non-lactose fermenting colony to be further analyzed may be too high [4]. River water is a major source of microbial pathogens in developing countries. River Narmada is the largest west flowing river of the Indian peninsula. The present study was designed to isolate *Shigella* strains from water samples of river Narmada in India by combination of PCR and culture methods and along with characterization through appropriate biochemical and serological tests. Furthermore, molecular techniques were used to genetically characterize such environmental strains and compare them with the clinical standard. These strains were also tested for the presence of *ipaBCD*, *ipaH* and *stx1* genes.

Materials and methods

Bacterial strains and water samples

Water samples were collected from 11 stations of the river Narmada (Amarkantak, Dindori, Mandla, Jabalpur, Narsinghpur, Hoshangabad, Omkareshwar, Koral, Neelkantheshwar, Ankaleshwar, Dahej), from July 2005 to June 2006 and brought to the laboratory under ice-cold conditions, concentrated on 47 mm diameter filters with 0.22 µm pore size, enriched in nutrient broth and incubated at 37°C for 6 to 8 h under shaking conditions [7]. Bacterial colonies were isolated on MacConkey agar medium, xylose lysine decarboxylate agar medium (XLD) and Hektoen enteric agar medium (HE) (Himedia, India). Non-lactose-fermenting colonies were picked up from culture plates and subjected to further analysis for the identification of possible *Shigella* colonies. *S. dysenteriae* type 1 strain BLSG/001, containing plasmid associated virulence genes (viz. *ipaH*, *ipaBCD* and *stx1*) obtained from National Institute of Cholera and Enteric Diseases (NICED), Kolkata, India, was used as the standard strain. After the identification, these isolates were maintained in the Bacteriology Lab, Department of Biological Sciences, R.D. University, Jabalpur (M.P.), India and were given Bacterial Germplasm Collection Centre (BGCC) numbers.

Phenotypic characterization

Biochemical and serological analysis

All the isolates were subjected for the following biochemical analysis: arginine dehydrolase, lysine decarboxylase,

ornithine decarboxylase, citrate utilization, indole production, oxidase production, urease production, methyl red test, voges-proskauer test, gluconate utilization, malonate utilization, and acid production from arabinose, dulcitol, glucose, lactose, maltose, mannitol, raffinose, rhamnose, sorbitol, sucrose and xylose [8]. On the basis of biochemical characteristics the isolates were identified with the help of Bergey's Manual of Systematic Bacteriology [9] and probabilistic identification of bacteria (PIB) computer kit [10]. Forty isolates presumptively confirmed on the basis of biochemical tests as *Shigella* spp. were subjected to serotyping by slide agglutination test using poly A, poly B, C1, C2, C3 and D antigens [11].

Antibiogram analysis

Antibiotic resistance/sensitivity profile was analyzed following disk diffusion method [15] using commercially available antibiotic disks (amikacin 30 µg, amoxicillin 30 µg, ampicillin 10 µg, cephalexin 30 µg, ceftazidime 30 µg, chloramphenicol 30 µg, ciprofloxacin 30 µg, gentamicin 10 µg, nalidixic acid 30 µg, norfloxacin 10 µg, streptomycin 25 µg, tetracycline 30 µg and trimethoprim 10 µg) from Himedia, India.

Genotypic characterization

Random amplified polymorphic DNA analysis (RAPD) and amplified ribosomal DNA restriction analysis (ARDRA):

For RAPD, genomic DNA was extracted as described by Sambrook [12]. Briefly, genomic DNA was prepared from bacterial cultures grown overnight in Luria Broth at 37°C by pelleting 1.5 ml culture by centrifugation at 8,000 rpm for 10 min, resuspending the pellet in 500 µl of sterile distilled water and boiling at 100°C for 10 min. It was then snap cooled at –20°C for 5 min, centrifuged at 5,000 rpm for 10 min at 4°C. The supernatant containing DNA template was amplified using (5'-CAT TCG ACC 3') primer [13]. The reaction was performed in a total volume of 25 µl per tube containing 2.5 µl of 10X PCR buffer [500 mM KCl, 100 mM Tris (pH 8.3), 25 mM MgCl₂], 3 µl of template DNA, 2 µl primer, 0.3 µl of Taq DNA polymerase (Bangalore Genei, India), and 2.5 µl of 2.5 mM each dNTP (dATP, dCTP, dGTP and dTTP). A negative control was assayed containing all components except template DNA. After initial denaturation at 94°C for 3 min the reaction mixture was run through 45 cycles of denaturation at 94°C for 1 min, annealing at 36°C for 1 min and extension at 72°C for 2 min, followed by a 10 min final extension period at 72°C. The expected size of the amplicons was ascertained by electrophoresis in 1.5% agarose gel with an appropriate molecular size marker (1 Kb DNA ladder, Bangalore Genei, India).

For ARDRA, genomic DNA was extracted as described above. PCR oligonucleotides primers were derived from conserved regions present at the edges of the 16S rDNA of genus *Shigella*. The sequences of the genus specific primers were “F – 5 AGA CTG CTA CGG GAG GCA GCA GT 3 and R – 5 GTT GCG CTC GTT GCG GGA CTT AA 3” [14]. 1 µl (around 10 ng) of the template DNA was added to 49 µl aliquots of the PCR mixture containing 5 µl of 10X buffer [500 mM KCl, 100 mM Tris (pH 8.3), 25 mM MgCl₂], 2.5 µl of each forward and reverse primer from a 10 picomol stock (i.e. 12.5 picomol of each primer), 2.5 µl of 10 mM (each) deoxynucleotide triphosphates and 0.5 µl of DNA polymerase (3 unit/µl) and the final volume was made 50 µl by adding sterile distilled water. After initial denaturation at 94°C for 3 min, the reaction mixture was run through 35 cycles of denaturation at 94°C for 1 min, annealing at 57°C for 1 min and extension at 72°C for 1 min, finally a 10 min extension period at 72°C was carried out. The 16S rRNA type was determined by digestion of the amplicons (1500 bp) with *HindIII* (A/AGCTT), *AluI* (AG/CT) and *EcoRI* (G/AATTC) (Bangalore Genei, India) and analyzed using 1% agarose gel with 0.5 µg/ml ethidium bromide.

Detection of virulence genes

A multiplex PCR assay for the genes encoding invasive plasmid antigens (*ipaBCD*) and Shiga toxin (*stxI*), as well as a second PCR assay for *ipaH* was used to detect the presence of these virulence genes in *Shigella* isolates using primer sequence for *ipaH* (F 5 - GCTGGAAAACT-CAGTGCCT-3, R 5 - CCAGTCCGTAAATTCATTCT-3), *ipaBCD* (F 5 - GCTATAGCAGTGACATG-3, R 5 - AC-GAGTTCGAAGCACTC-3) and *stxI* (F 5 - CAACACTG-GATGATCTCAG-3, R 5 - CCCCCTCAACTGCTAATA-3) [7].

Results

Biochemical and serological analysis

Out of 40 isolates of *Shigella* spp., 7 were identified as *S. dysenteriae*, 23 were identified as *S. flexneri* and 10 were identified as *S. sonnei* on the basis of morphological, cultural and biochemical characteristics. All isolates showed different agglutination result. Twenty-three isolates of *Shigella* showed agglutination with polyB antigen and were identified as *S. flexneri*. 7 isolates of *Shigella* showed agglutination with polyA antigen and were identified as *S. dysenteriae* and 10 isolates of *Shigella* showed agglutination with D antigen and were identified as *S. sonnei*.

Antibiogram analysis

The antibiogram analysis of *Shigella* spp. showed that out of 7 isolates of *S. dysenteriae*, 71.4% of the strains were resistant to amoxicillin, 57% each to gentamycin and cephalexin, 42.8% each to amikacin and nalidixic acid, 28.5% each to ampicillin and streptomycin, 14.3% to ceftazidime, ciprofloxacin, tetracycline and norfloxacin. All the strains were susceptible to chloramphenicol and trimethoprim. Likewise, out of 10 isolates of *S. sonnei*, 70% were resistant to amoxicillin, 60% to ampicillin, 40% each to nalidixic acid, gentamycin and tetracycline and 10% to trimethoprim, but all the strains were susceptible to amikacin, cephalexin, ceftazidime, chloramphenicol, ciprofloxacin, norfloxacin and streptomycin. Out of 23 isolates of *S. flexneri*, 52.5% strains showed resistance to ampicillin, 43% to amoxicillin, 34.8% ceftazidime 30% to nalidixic acid, 26% each to tetracycline and trimethoprim, 17.4% each to streptomycin, chloramphenicol and gentamycin while all the isolates were susceptible to amikacin, cephalexin, ciprofloxacin and norfloxacin (Table 1).

RAPD and ARDRA of *Shigella*

Analysis of all 40 isolates revealed that the *Shigella* species produced five different RAPD pattern with bands ranging from 5–11. One common band of 500 bp was observed in all the isolates, while a band of 250 bp was evident in all the isolates except for BGCC# 384, 387, 390, 399, 410, 413, 414 and 419. 23 isolates of *S. flexneri* showed 3 RAPD profiles with bands ranging from 7–11, 10 isolates of *S. sonnei* showed 2 RAPD profiles with bands ranging from 7 to 9 and 7 isolates of *S. dysenteriae* showed single RAPD profile with 7 bands (Fig. 1). All isolates were analyzed by unweighted pair group method with arithmetic mean (UPGMA) on the basis of RAPD profile. Total two clusters (A and B) were found in the dendrogram at a coefficient level of 3.36. Cluster A was represented by 9 isolates and B was represented by 31 isolates. Cluster B was further divided into B1 and B2 at coefficient level of 3.16.

The 16S rRNA gene was enzymatically amplified for all the 40 isolates belonging to 3 described genomic species of the genus *Shigella*, and the amplicon was digested independently with 3 different restriction enzymes (*EcoRI*, *HindIII* and *AluI*). All the 3 enzymes produced 2–5 fragments on digestion. However, *EcoRI* resulted in overall higher dissimilarity among the isolates (Fig. 2). Based on *EcoRI* digestion two major divisions designated as A and B were found with a dissimilarity coefficient of 2.30 on UPGMA cluster analysis. The majority of *Shigella* strains were found to be in group B, which was further divided into subdivision B1 and B2 (Data for *HindIII* and *AluI* not shown).

Table 1 Patterns of antimicrobial resistance in *Shigella* isolates

Antibiotic resistance pattern	No. of <i>Shigella</i> isolates (n=40)		
	<i>S. flexneri</i> (n=23)	<i>S. dysenteriae</i> (n=07)	<i>S. sonnei</i> (n=10)
A, Am, Cae, Na, Ce, G, Cf, T, S, Ak	00	01	00
A, Am, Cae, Na	02	00	00
A, Am, Na, Tr	00	00	01
A, Am, Cae, S	01	00	00
Cae, Na, S	02	00	00
G, T, Na	00	00	03
A, Am, G	02	00	00
Am, G, Ce	00	02	00
G, T, Am	00	00	01
Na, S, Ce	00	01	00
A, Am, Tr	01	00	00
A, Am, Ak	00	01	00
Na, Ak, Nx	00	01	00
Am, G	00	01	00
T, Tr	03	00	00
A, Am	04	00	05
A, Tr	02	00	00
Cae, Na	03	00	00
C, T	03	00	00

Ak (Amikacin), Am (Amoxicillin), A (Ampicillin), Ce (Cephotaxime), Cae (Ceftazidime), C (Chloramphenicol), G (Gentamycin), Na (Nalidixic acid), Nx (Norfloxacin), S (Streptomycin), T (Tetracycline) and Tr (Trimethoprim).

Lane 1. BGCC382, 4.BGCC383, 6.BGCC385, 7.BGCC389, 9.BGCC391, 10.BGCC392, 12.BGCC396, 15.BGCC394, 16.BGCC395, 17.BGCC397, 22.BGCC404, 24.BGCC398, 25.BGCC402, 26.BGCC406, 29.BGCC408, 30.BGCC407, 31.BGCC409, 32.BGCC411, 36.BGCC418, 38.BGCC412, 39.BGCC416, 40.BGCC421, 41.BGCC419, (*S. flexneri*),
 Lane- 3. BGCC386, 5.BGCC388, 8.BGCC393, 11.BGCC400, 13.BGCC401, 18.BGCC403, 19. BGCC405, 28.BGCC415, 33.BGCC417, 34.BGCC420, (*S. sonnei*),
 Lane 2. BGCC384, 14.BGCC387, 20.BGCC390, 23.BGCC399, 27.BGCC410, 35.BGCC413, 37.BGCC414, (*S. dysenteriae*), and Lane- 21 (control, *S. dysenteriae* type 1)
 M- DNA ladder 1 kb marker

Fig. 1 RAPD profile of *Shigella* spp. isolated from river Narmada

PCR analysis of virulence genes

The protein product of *IpaH* gene is necessary for the invasion of colonic epithelial cells and also for the detection of *Shigella* in the environment. During the present investigation all the 40 isolates were subjected to PCR for the detection of the *IpaH* gene, the *IpaBCD* gene and the shiga toxin (*stx1*) gene. The invasive gene *ipaH* (427 bp) was present in all the 40 isolates confirming the presence of *Shigella* in river water (Fig. 3). The *ipaBCD* gene (500bp) was present in all the 23 isolates of *S. flexneri* while it was absent in all the isolates of *S. dysenteriae* and *S. sonnei*. The shiga toxin (*stx1*) gene was present in all the 7 isolates of *S. dysenteriae*

but absent in all the isolates of *S. flexneri* and *S. sonnei* (Fig. 4).

Discussion

Understanding the ecology of *Shigella* had been limited mainly due to the lack of suitable techniques to detect the presence of *Shigella* in environment samples [7]. In the present study we used molecular techniques as well as conventional typing methods including biotyping and serotyping to detect *Shigella* in river water with special reference to its virulence genes and antibiogram analysis.

Lane 1, 2, 4, 8, 10, 11, 13, 14, 15, 16, 17, 22, 24, 26, 27, 28, 29, 31, 32, 36, 38, 39, 41 (*S. flexneri*), Lane 5, 7, 12, 19, 20, 23, 25, 35, 37, 40 (*S. sonnei*), Lane- 3, 6, 9, 18, 30, 33, 34 (*S. dysenteriae*), and Lane –21 (Control- *S. dysenteriae* type 1), M- DNA ladder 1 kb marker.

Fig. 2 ARDRA profile of *Shigella* spp. isolated from river Narmada

Lane 1, 2, 4, 8, 10, 11, 13, 14, 15, 16, 17, 22, 24, 26, 27, 28, 29, 31, 32, 36, 38, 39, 41 (*S. flexneri*), Lane- 5, 7, 12, 19, 20, 23, 25, 35, 37, 40 (*S. sonnei*), Lane- 3, 6, 9, 18, 30, 33, 34 (*S. dysenteriae*), and Lane –21 (Control- *S. dysenteriae* type 1), M- DNA ladder 1 kb marker.

Fig. 3 Uniplex PCR analysis of *IpaH* gene of *Shigella* spp. isolated from river Narmada

Fig. 4 Multiplex PCR analysis of *IpaBCD* and *Stx 1* gene of *Shigella* spp. isolated from river Narmada

We standardized the assay by culturing the environmental water samples and simultaneously conducted PCR tests. In a similar study, the *ipaH* gene was used as an indicator to detect the presence of *Shigella* in environmental waters [7]. In our study the isolates were assayed for both the presence of *ipaH* gene and the plasmid-associated virulence genes including *ipaBCD* and *stx1* genes. In developing countries, where sanitation is poor, fresh contamination of surface water by fecal material of dysentery patients may result in the presence of *Shigella* in surface water. Several previous studies have also detected *Shigella* in surface waters or sewage samples [7, 16] and have indicated that *Shigella* strains can be transported by surface waters [17]. However, in the present study the absence or deletion of crucial virulence genes in environmental *Shigella* isolates suggested that the presence of these strains in river water may not be due to fresh fecal contamination of river water, but instead, these strains have survived and persisted in the aquatic environment. It appears that since in the environment the invasive genes do not have any known function, the bacteria apparently lost part of the plasmid-encoded genes. However, a chromosomally located multicopy virulence gene, *ipaH*, which is known to have a role in producing invasive characteristics [7] was found to be more stable and was present in all the environmental strains analyzed. Therefore, PCR screening of these strains for *ipaH* genes proves to be a better indicator for the presence of *Shigella* than screening for the plasmid encoded *ipaBCD* genes.

The presence of *Shigella* in river waters, as demonstrated in the present study, may have public health implications.

Although most of the environmental strains lacked one or more of the known virulence genes, the environmental strains shared an ARDRA type with standard strain and belonged to the three species, which are associated with most of the cases of Shigellosis in India and many other developing countries. The detection of *Shigella* strains in river water thus appears significant. In the present investigation, ARDRA with a probe complementary to 16S rRNA was performed. Unlike the ARDRA, where the differentiation was species specific, in RAPD the isolates were found to be distributed in all the clusters, depending on the banding pattern. This observation establishes the superiority of ARDRA over RAPD in establishing the genetic differentiation among fresh water isolates of the river Narmada. ARDRA and RAPD-PCR techniques have been used for the typing of bacteria in epidemiological and ecological studies and their advantage and disadvantages are well known. The rRNA-coding regions, notably 16S rDNA used extensively underpin phylogenetic structures at the species level [18]. RAPD or AP-PCR (arbitrarily primed polymerase chain reaction) is a DNA fingerprinting technique, which is one of the most sensitive and efficient method for distinguishing different strains of a species [19]. RAPD was chosen as it has been reported to be an effective subtyping method for several other species, namely *Listeria*, *Yersinia* [20], *Staphylococcus* [21], *Streptococcus* [22] and *Aeromonas* [23]. Antimicrobial resistance in enteric pathogens is of great significance in the developing world, where the rate of diarrheal diseases is highest. The progressive increase in antimicrobial resistance among enteric patho-

gens in developing countries is becoming a critical area of concern.

During the present investigation it was observed that most of the isolates were resistant to amoxicillin while variable resistance patterns were observed with different species of *Shigella* with respect to different antibiotics. Moreover, many studies have been carried out almost exclusively with clinical strains; while there are only few studies on the pathogenic aspects of environmental strains. Since the aquatic environment is implicated as the reservoir for these microorganisms and consequently responsible for their transmission in humans, it is obvious that detailed studies on the pathogenic potential of the environmental *Shigella* strains will certainly contribute to the understanding of virulence properties of these bacteria.

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