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Nalidixic acid resistant *Shigella sonnei* infection among dysenteric children in Port Blair

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Shigellosis is very common in Andaman and Nicobar Islands, India. Two major species and serotypes, *Shigella flexneriae* 2a and *Shigella dysenteriae* type 1 were the cause of shigellosis in the Island till 2001. During 2002 to 2003, the scenario of shigellosis shifted and *Shigella sonnei* emerged as the prime and sole pathogen. On examination of 106 diarrhoeic stool samples, a total of seven *S. sonnei* was isolated. All the isolates were nalidixic resistant. The shift of predominant nalidixic acid-resistant trait of *S. dysenteriae* type 1 to *S. sonnei* had been observed in this study and was reported elsewhere (Ghosh et al., 2003). The present study aimed to study genotypic characterization of the nalidixic acid resistant *S. sonnei*. The molecular typing of isolated strains was carried out by plasmid profiling, random amplified polymorphic DNA (RAPD) and pulsed field gel electrophoresis (PFGE). The results revealed the clonality of the nalidixic acid-resistant *S. sonnei*. The study explores a note of caution to the public health for an epidemic due to nalidixic acid resistant *S. sonnei* in near future.

Key words: Nalidixic acid resistance, *Shigella sonnei*, dysenteric children.

INTRODUCTION

Antimicrobial resistance is a crucial determinant for causing the persistent infection, which warrants epidemics too. Shigellosis is still a perpetual problem in many parts of tropical countries including India. It remained endemic in Andaman and Nicobar Islands, since an early epidemic occurred due to *Shigella dysenteriae* type 1 and since scientific attempts were initiated to examine the diarrhoeic hospitalized patients during 1994 to 2003 (Sen et al., 1986; Ghosh and Sehgal, 1996, 1998). The pattern of *Shigella* infection had been shifting since the first epidemic to pre-tsunami era.

Like in mainland India and most of the other developing countries *Shigella flexneri* 2a has been the commonest isolate with the proportion of different species and serotypes of *Shigella* isolated showing considerable variation (Niyogi, 2005). Antibiotic resistance pattern also showed variations. As in all developing countries, the

choice of limited number of effective antimicrobials for empirical treatment of suspected infections caused by rapid emergence of multi-drug resistant Shigellae has been a problem to clinicians in these islands. The ampicillin, co-trimoxazole resistant strain of *S. dysenteriae* type 1 that was widespread in mainland India during 1980s, was first observed in the Andaman islands in 1986 (Sen et al., 1986) which prompted clinicians to discontinue the use of these antimicrobials and by early 1990s, nalidixic acid became the drug of choice in treatment. By 1994, when routine surveillance started, all Shigellae isolated became resistant to ampicillin and 80.6% to cotrimoxazole (Ghosh and Sehgal, 1996). Nalidixic acid resistance (15.1%) was first observed in *S. dysenteriae* 1 during an outbreak of diarrhoea in 1995 (Ghosh and Sehgal, 1998) but it remained confined to *S. dysenteriae* and occasional *S. flexneri* isolated from sporadic cases till 1999/2000. However, *S. sonnei* were isolated simultaneously and found sensitive to nalidixic acid and to other quinolones till 2000 (Ghosh et al., 2003).

Emergence of multiple drug resistance among strains of *S. dysenteriae* type 1 from ampicillin to sulphamethoxazole-trimethoprim, nalidixic acid and to

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ciprofloxacin and subsequently among other members of the genus had been observed in many countries (Sack et al., 2001). The most virulent serotype of *S. dysenteriae* type 1 has been responsible for large dysentery epidemics in India, Guatemala and other parts of Central America, Zaire, Kenya, Bangladesh, and West Africa (Niyogi, 2005). The similar dynamism had been also maintained among the islands' isolates of *Shigellae*. The shift of antimicrobial resistance and predominance was striking in the present study. Emergence of *S. sonnei* as a major cause of shigellosis with the emergence of nalidixic acid resistance property brought a paradigm shift in shigella infection in island (2003).

During 2003 to 2009, the shigella infection was dropped drastically in the island. However, resistance to other fluoro-quinolones (like, ciprofloxacin, norfloxacin, gratifloxacin) emerged subsequently (Roy et al., 2010).

In this report, the emerged nalidixic acid-resistant *S. sonnei* were analysed using molecular typing tools like plasmid profile, random amplified polymorphic DNA (RAPD) and pulsed field gel electrophoresis (PFGE) and compared with the sensitive strains with an objective of looking into their clonal relationship.

MATERIALS AND METHODS

Bacteriological analyses

A total of 106 faecal samples, representing individual subject, were collected from acute diarrhoeic paediatric patients admitted in GB Pant Hospital, Port Blair, during May 2001 to 2002. Specimens were processed and examined following standard microbiological procedure as described elsewhere (Ghosh and Sehgal, 1996, 1998); using three different plating media- MacConkey agar (Oxoid, UK), Hektoen Enteric agar (Oxoid, UK) and Desoxycholate Citrate agar (Oxoid, UK).

Prospective *Shigella* colonies were subjected to bio-typing by indole test (1% bacto-peptone, (Oxoid, UK)); mannitol utilization test, motility test and nitrate reduction test (Mannitol motility medium, HIMEDIA, India); citrate utilization test, urea hydrolysis test, catalase and oxidase test etc. Microscopic examination had also been carried out for polymorpho-nuclear leukocytes (PMNLs), red blood cells (RBCs) epithelia etc. before the microbiological analyses.

Seven strains from 106 samples and another nine strains from other studies (3 from community based studies; PP-57, PP-84, PP-374 isolated during 1999 to 2001 and randomly selected 6 from culture bank, isolated during 1994 to 1999 from hospitalized paediatric patients)- as a whole total of 16 strains were included in this study.

Serotyping

Serotyping was carried out by slide agglutination method and was identified using commercially available standard antisera (Denka Seiken Co Ltd., Tokyo, Japan). In every case, normal saline was used before using the type-sera (Ghosh and Sehgal, 1996, 1998).

Antibiotic susceptibility testing

Standard procedure was followed to examine the antibiotic susceptibility of isolated strains (Bauer et al., 1966; National

Committee for Clinical Laboratory Standards, 1993), using 8 different antibiotic discs (HIMEDIA, India; µg/disc) like ampicillin (AMP, 10); chloramphenicol (CHL, 30); ciprofloxacin (CIP, 5), Co-trimoxazole (CoT, 30), Gentamicin (GEN, 10), Nalidixic acid (NAL, 30), Norfloxacin (NOF, 10), and Nitrofurantoin (NIT, 300) respectively. E-test (AB BIODISK, Sweden) and minimum inhibitory concentration (MIC) were also carried out accordingly (National Committee for Clinical Laboratory Standards, 1993).

Plasmid profile analysis

A total of 16 *S. sonnei* strains (nine -resistant and seven- sensitive to nalidixic acid) was grown in Luria broth (Difco, USA) overnight at 37°C and plasmids were prepared by the simplified alkaline lysis method (Kado and Liu, 1981). Plasmid DNA was detected by agarose gel electrophoresis in 1.0% agarose with 0.5 µg/ml of ethidium bromide and photographed with UV illumination using gel documentation system (Viber Lourmat, Germany). Suitable DNA marker (1 kb, Sigma Chemicals, USA) and DNA from *E. coli* V517 were used in each gel. Only small plasmid bands, which appeared as bright and below the chromosomal band, were examined. Large plasmids were not considered because of their instability.

RAPD analysis

The bacterial cultures grown for plasmid analysis were also used for RAPD analysis. In brief, bacterial cells were harvested from overnight culture in Luria broth and were treated with 10 mg/ml of lysozyme (Sigma Chemicals, USA) for 2 h at 37°C, followed by successive treatments with 10% SDS, 10 mg/ml of proteinase K (Sigma Chemicals, USA) , 5 M NaCl and CTAB/ NaCl. DNA was extracted using chloroform: Isoamyl alcohol (24:1) and precipitated with chilled ethanol. The extracted DNA was dissolved in Milli Q water and concentration was estimated at 260/280 nm using UV-Vis spectrophotometer (Biorad, USA).

The primer used with the sequence 5' AAA GAA GGA CTC AGC GAC TGC G 3' (S6) - was chosen for generation of RAPD fingerprints for each strain. PCR (polymerase chain reaction) was performed at least twice in DNA Engine PTC200 (MJ Research Inc., USA) in 50 µl reaction volume with 50 ng purified DNA, 2 µM primer, 250 µM of each dNTP, 15 mM MgCl₂, 0.5 U of Taq polymerase in 10 mM Tris-HCl (pH 9.0) and 50 mM KCl. The temperature program consisted of 1 cycle of 3 min at 97°C, 1 min at 40°C, and 1 min at 72°C; 4 cycles of 1 min at 97°C, 1 min at 40°C, and 1 min at 72°C; 24 cycles of 1 min at 95°C, 1 min at 55°C and 1 min at 55°C, and 7 min at 72°C. Reaction products were electrophoresed on 20 cm long 1% agarose gel, stained with 0.5 µg /ml ethidium bromide , viewed and photographed with UV illumination using gel documentation system (Viber Lourmat, Germany). Suitable DNA marker (1 kb, Sigma Chemicals, USA) and DNA from *E. coli* V517 were used in each gel. Dendrogram was generated using Bioprofit software.

Pulsed field gel electrophoresis (PFGE)

All the strains as shown in Table 1 and two sensitive strains (as in lane 11 and 12 of plasmid analysis) were subjected for PFGE analysis. In brief, PFGE agarose plugs of the genomic DNA were prepared as described previously (Yamasaki et al., 1997) with brief modifications. Single colony was selected and was inoculated into a 5 ml Luria Broth in shake culture at 37°C. The bacterial culture was spread onto Luria agar plate and was incubated for overnight at 37°C. The bacterial suspension, prepared by harvesting bacterial colonies from LA plate into 1 M TE buffer (pH: 7.2), was adjusted to spectrophotometer (Smartspect, Bio-Rad, USA) reading of 1.35 to

Table 1. Distribution of sensitive and resistant strains of *S. sonnei* as isolated from time to time*.

S/N	Strain no.	Age (Months)/ sex (M/F)	Stool character	Date of isolation	Location/ patients' home	Resistant pattern
1	DS-103	72/M	Watery with mucus	15.03.2002	Mini Bay	AMP, CoT, NAL
2	DS-100	18/M	Watery with mucus	13.03.2002	Nayagaon	AMP, CoT, NAL, NIF
3	DS-65	12/M	Watery with mucus	29.08.2001	Junglighat	AMP, CoT, NAL
4	DS-64	4/F	Watery with mucus	29.08.2001	Junglighat	AMP, CoT, NAL
5	DS-62	24/M	Watery with mucus	09.08.2001	Premnagar	AMP, CoT, NAL, CHL
6	DS-61	16/F	Watery with mucus	31.07.2001	Brichgunj	AMP, CoT, NAL
7	DS-46	36/M	Semisolid with mucus	27.06.2001	Shadipur	AMP, CoT, NAL, NIF
8	PP-374	72/M	Semisolid	04.07.2000	Protherapur	AMP, CoT, NAL
9	PP-84	15/F	Watery with mucus	29.09.1999	Protherapur	AMP, CoT, NAL
10	PP-57	35Y/F	Watery with mucus	18.09.1999	Protherapur	AMP, CoT

*Serial no. 1 to 7 are from hospitalized patients and serial no. 8 to 10 are from community patients.

1.40 at 620 nm ($\sim 10^9$ CFU/ml). A 200 μ l of such bacterial suspension with 10 μ l of proteinase K (20 mg/ml) was then mixed with an equal volume of 2% low melting agarose (Bio-Rad, USA), dispensed in a plug-mold (Bio-Rad, USA) and allowed to solidify at room temperature. For lysis, the resulting plugs were placed in a mixture of (10 mM Tris-HCL, pH 7.2; NaCl, sodium desoxycholate and sodium-lauryl sarcosine and proteinase K). Following 2 h incubation in a water bath adjusted to 54°C with mild shaking. Plugs were washed twice with sterile water, pre-heated at 50°C for 30 min for four times for 15 min each. Each plug was pre-incubated in respective restriction enzyme digestion buffer for 1 h at 37°C. A slice of each plug was cut and incubated overnight with 30U of *NotI* (New England Biologic, UK).

The PFGE of *NotI* digested inserts were carried out in a contour-clamped homogeneous electric field on a CHEF Mapper system (Bio-Rad, USA) using 1% PFGE grade agarose in 0.5x TBE (44.5mM Tris-HCl, 44.5 mM boric acid, 1.0 mM EDTA, pH 8.0) for 40.24 h at 14°C with an electric field of 6 V/cm. Run conditions were generated by the auto algorithm mode of the PFGE system based on the DNA size range 20 to 300 kb. A lambda ladder (Bio-Rad, USA) was used as the molecular mass standard. After electrophoresis, the gel was stained in etidium bromide (0.5 μ g/ml) for 30 min and destained in MQ water for 1 h. DNA bands were visualized and photographed under UV light using Viber Lourmat gel documentation system.

RESULTS

A total of one hundred and six faecal samples were examined which yielded 8.49% (Nine isolates) of Shigellae. Among all, seven were of *S. sonnei* (phase 1) and one each was *S. flexneri* 3a and *S. flexneri* 4b, respectively. All the strains were isolated from children below five years with one patient of 4 months old and other one was of 35 years old (Table 1). They had abdominal pain, watery stools with mucous and fever.

The antibiogram of all the isolates was presented in Table 1. The result of antibiotic sensitivity showed the predominance and emergence of nalidixic acid resistance among *S. sonnei*. All the seven *S. sonnei* isolates were resistant to nalidixic acid, ampicillin and co-trimoxazole. Other Shigellae isolates were sensitive to nalidixic

acid. The nalidixic acid resistant strains emerged among *S. sonnei* isolates since 29.09.1999 (Table 1). MIC of nalidixic acid was estimated by using e-test, ranged between 24 to 96 μ g/ml (mean: 40.88 ± 26.06) (Ghosh et al., 2003).

The comparative plasmid profiles between nalidixic acid resistant (*nal^r*) and nalidixic acid sensitive (*nal^s*) *S. sonnei* showed that the *nal^r*-strains are of similar pattern and *nal^s* are of variable patterns (Figure 1). The similar patterns among the *nal^r* strains also envisage the clonal character among the isolates. As has been shown in the Figure 1, each of the *nal^r*-strains had seven plasmids and none of them possessed the large plasmid. All the nalidixic acid resistant strains (Lane 1-9) of *S. sonnei* showed similar pattern with 5 prominent plasmids range between 1 to 10 kb (L1-L9) in comparison to sensitive strains (L10-L16) with 120 to 1kb plasmids of variable numbers. The L-10 represents the *nal^s*-strain which was isolated from community.

RAPD and PFGE profile of both resistant strains showed highest degree of similarity and homology as has been expressed by dendrograms (Figures 2 to 5). Sensitive strains showed varied display among themselves and with resistant cluster. This analysis revealed that the nalidixic acid resistant *S. sonnei* comprised a single cluster and thus clonal.

DISCUSSION

Shigella infection is a very common diarrhoeal episode among the islanders of Andaman and Nicobar Islands. The infection is found endemic and unique at this part of geography. Importance to this study is associated with the emergence of new species with nalidixic acid resistance, which is a rare occurrence. The changing patterns of *Shigella* infection found in different parts of India (Dutta et al., 2002; Pazhani et al., 2005; Taneja, 2007) and elsewhere (Farshad et al., 2006). The infection

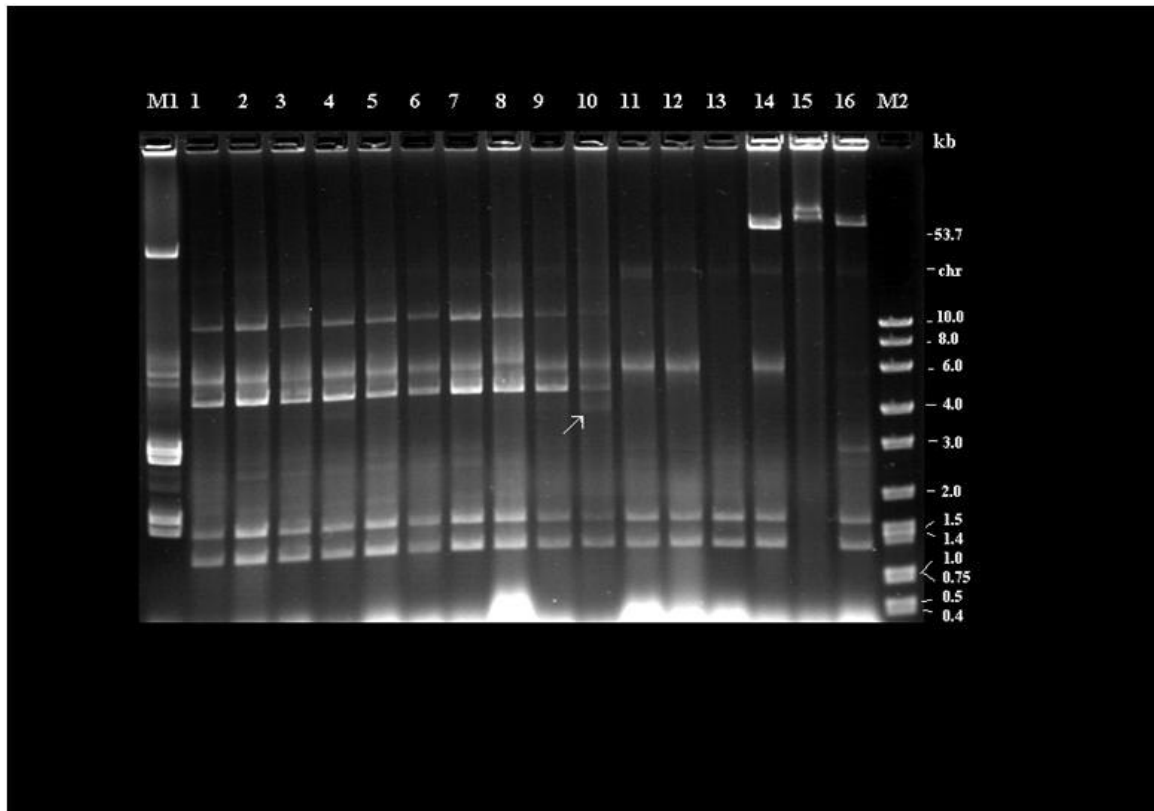


Figure 1. Plasmid profile of nalidixic acid resistant (Lane 1-9) and nalidixic acid sensitive (Lane 10 - 16) *S. sonnei* with M1 (V517) and M2 (1 kb ladder, Sigma chemicals, USA), the molecular weight determining markers. All the nalidixic acid resistant strains of *S. sonnei* showed similar pattern with 5 prominent plasmids range between 1 and 10 kb (L1 to L9) in comparison to sensitive strains (L10 to L16) with 120 to 1 kb plasmids of variable numbers.

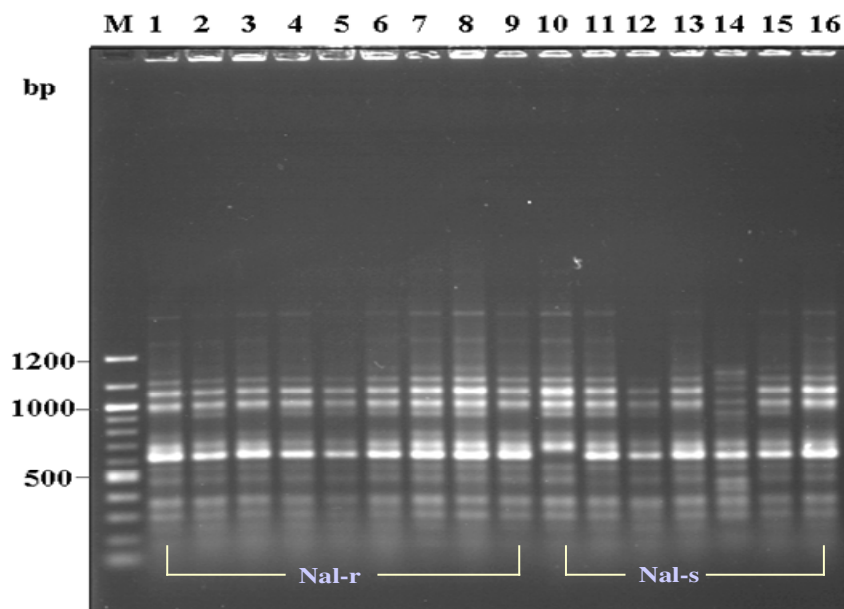


Figure 2. RAPD profile of nal-*r* and nal-*s* *S. sonnei*. Lanes 1 to 9 are of nalidixic acid resistant and Lanes 10 to 16 of nalidixic acid sensitive *S. sonnei* with M, the molecular weight determining marker (100 bp). Profile does not show discrepancy between two groups of strains.

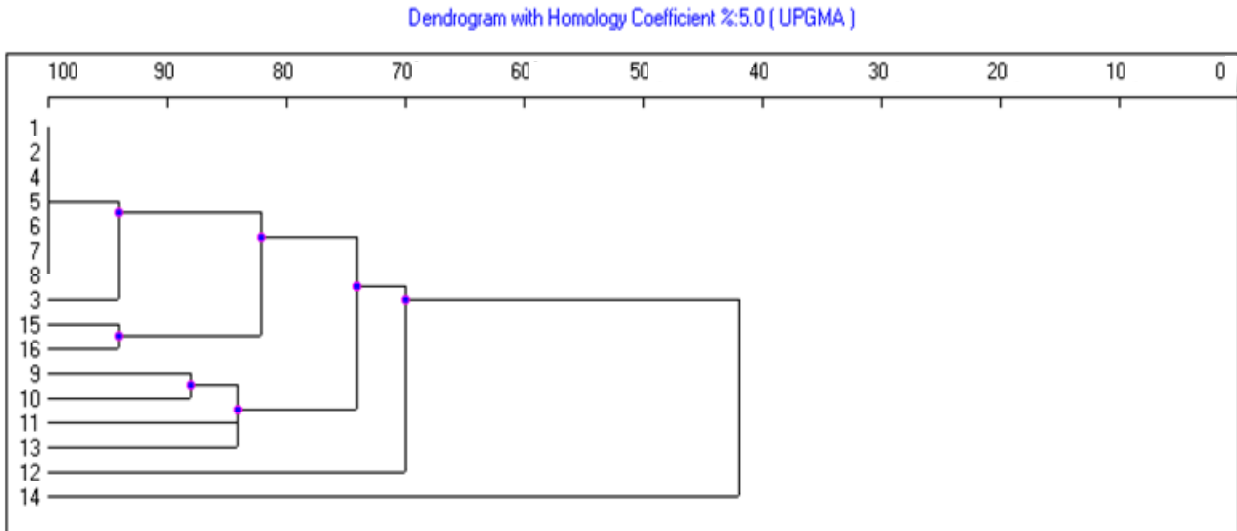


Figure 3. The dendrogram clearly represents the clonality of 7 strains of 9 with 100% homology and with negligible distance from other two strains (#3 and #9) with >85% homology among nalidixic acid resistant. It is explicable that the sensitive strains are of different stalks with variable homology (strains 10 to 16).

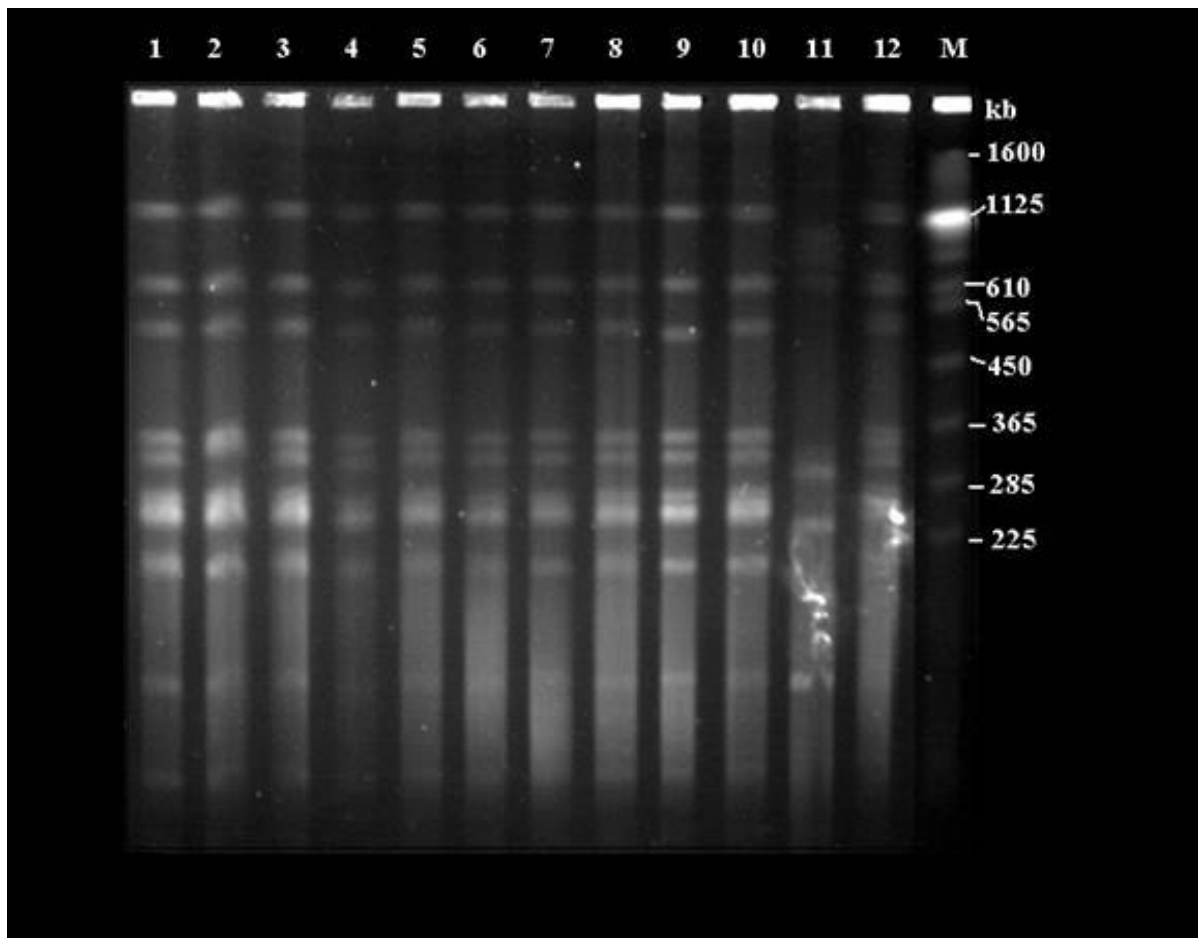


Figure 4. The PFGE profile of nalidixic acid resistant and sensitive *S. sonnei*. This figure depicts the profile differences between two groups (Lane 1 to 9 versus 10 to 12). Resistant strains show the highest homology among them with 10 bands with equal distribution. In contrast, other strains show different with nalidixic acid sensitive phenotypes.

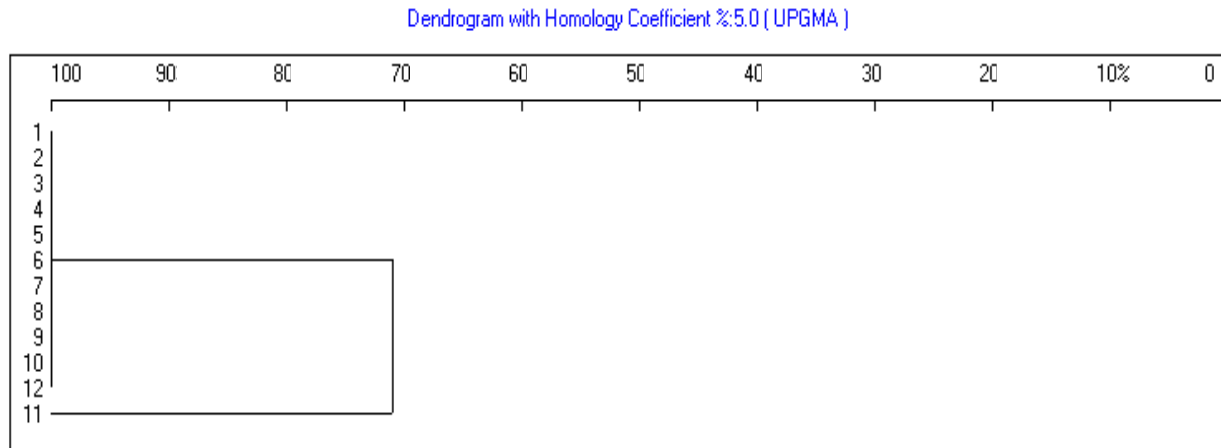


Figure 5. The dendrogram of PFGE profile among the resistant (stains 1 to 9) and sensitive (strains 10 to 12) to nalidixic acid - clearly represents the clonality with an exception of stain #11, irrespective of reaction to nalidixic acid. This may suggest the emergence of resistant stains maybe a case of point mutation and that maybe the case of showing 100% homology.

prevailed in this island because mainly of two major species and serotypes; *S. flexneri* 2a and *S. dysenteriae* 1. Similarly, the property of multiple drug resistance was seen among them. The history of shigella infection in the islands was also as usual till 2000 like elsewhere (Farshad et al., 2006; Ghosh and Sehgal, 1996; 1998). The prime shift in infection and emergence of nalidixic resistance was a circumventing event in island.

The alarmingly increasing and rapid emergence of antibiotic resistance among enteric pathogens like Shigellae, vibrios and campylobacter is majorly due to inappropriate use and easy availability of drugs in different parts of the world (Sack et al., 2001). The abuse/misuse of antibiotics in a closed ecosystem like Port Blair are easy to understand. There is only one referral hospital, GB pant Hospital with 30 beds in paediatric division. The antibiotics administration is controlled by the hospital authority following the national scheme. During 1994, when the author undertook a hospital based study on diarrhea, under the aegis of Regional Medical Research Centre, Port Blair, islanders were completely dependent on the hospital system for treatment. There was only one public medical shop (over-the-counter) for common people. Ampicillin and sulphamethoxazole-trimethoprim were the common antibiotics for the treatment of diarrhoea. The microbiological evidence showed that the majority of the episodes were *Shigella* associated and were resistant completely to ampicillin, but sensitive to ciprofloxacin, norfloxacin, gentamycin and nalidixic acid (Ghosh and Sehgal, 1996). During 1996, many shops opened and indiscriminate use of antibiotics eventuated. As a result, subsequent isolates were mostly multiply resistant with the emergence of nalidixic acid and gentamicine, as reported elsewhere (Ghosh et al., 2003). Apart from emergence of antibiotic resistance to indiscriminately used drugs, shifts were observed from

serotype to serotype (*S. dysenteriae* type 1 infection shifted to *S. flexneri* 2a and to *S. sonnei* during the study period).

The emergence of nalidixic acid resistance among *S. sonnei* is very much interesting in the south-east asian geography. As the islands- union territory of India is situated about 1200 km away from mainland and most of the human activities are centered at the capital town, Port Blair, so the introduction of new pathogen to the Island, ecosystem may not be ruled out. In a separate study, the exotic strains of *Vibrio cholerae* O1 caused cholera epidemic first time in the island (Roy et al., 2005; Sugunan et al., 2004). But the trend followed in shigellosis, it appears to be of islands'-origin, as shown by RAPD and PFGE result. Simultaneously, the isolation rate of *S. sonnei* (28%) has been increased in other parts of the country as found in Kolkata (East India); and in North India as well. During post epidemic and subsequent years, *S. sonnei* was the second predominant serotype studied in Kolkata (Dutta et al., 2002; Niyogi, 2005; Taneja, 2007) and a high rate of *S. sonnei* (70.39%) infection was reported in Iran (Farshad et al., 2006). The scenario of emergence of nalidixic acid resistance replacing major two serotypes in islands is an enigma and shows similarity in trends of shigella infection as seen in the industrialized countries (Farshad et al., 2006). The re-emergence of *S. dysenteriae* type 1 with multiple antibiotic resistances has been experienced in many tropical developing countries including mainland of India but emergence of *S. sonnei* with such virulence is a matter of concern.

Farshad et al. (2006) characterized Shigellae using plasmid profile and showed a variety of profiles even within the same serotype with a total of 42 genotypes. In the present study, all the resistant isolates showed similar pattern with seven plasmids, ranging between 10 to 1.2 kb

and thus shows a single genotype. Other *S. sonnei* isolates showed different patterns with large plasmid. Maybe the plasmid profile is a good analytical tool to reveal the spatial distribution of strains of the same community. Though, it may also be lost due to many reasons like long storage or/ and multiple subculturing.

However, the clonal character of the nalidixic acid resistant *S. sonnei* isolates has been restored. The last sensitive strain was isolated on 18.09.1999 (PP-57) and the first nal^r strain was isolated on 29.09.1999 (PP-84). However, the clonality has further been conformed by the RAPD and PFGE analysis. In between time, there occurred a great natural calamity, the tsunami in 2004. Subsequent report on diarrhea has been reduced. Emergence of fluoroquinolone resistance has been becoming a new public health threat (Roy et al., 2010). However, the molecular epidemiology of the studied Shigellae is a document of sustaining the clonality among *S. sonnei* in the islands.

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