

Characterization of Multiple Antimicrobial Resistant *Shigella sonnei* Isolated from Diarrhoeal Patients in Azad Kashmir, Pakistan*

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Abstract.- The antimicrobial susceptibility patterns for 83 *Shigella sonnei* isolated from diarrheal patients admitted to hospitals in Azad Kashmir Pakistan were analyzed from 1994 to 1998 to determine their changing trends in response to twenty antibiotics. The isolates showed highest resistance against penicillin (P) followed by carbenicillin (Ca), ampicillin (A), tetracycline, erythromycin, ceftizoxime, kanamycin, co-trimoxazole, piperacillin, amoxicillin, amikacin, streptomycin, nalidixic acid, gentamicin, chloramphenicol, cephalothin and ceftriaxone. All *S. sonnei* isolates were sensitive to cefixime, ciprofloxacin and enoxacin. Multiple drug resistance was observed in this study ranging from three to ten drugs and was resistant to three or more antibiotics at level as high as 300µg/ml. The resistant isolates showed different patterns of antibiotics resistance. The most common pattern was PCaA. The plasmids were observed in (29.2%) MDR strains of *S. sonnei* which were found resistant to three or more antibiotics. The number of plasmids varied from one to seven. Analysis of plasmid DNA of *S. sonnei* revealed that all the strains contained a heterogeneous population of plasmids ranging between >23.1 kb to <2.0 kb. Based on molecular weight, the pattern of different plasmids was also very diverse. Depending on the number of plasmids, individual strains were grouped into nine different plasmid patterns. The plasmids (>23.1 Kb and 23.1 Kb) could only confer ampicillin, chloramphenicol and sulfamethoxazole-trimethoprim resistance to the competent cells of *E. coli* HB101.

Key words: *S. sonnei*, antibiotic resistance, R-plasmid.

INTRODUCTION

Shigellosis is a major public health concern, over 163.2 million cases are reported in developing countries, and 1.5 million cases are reported in developed countries every year. The disease is highly contagious due to its low infection dose as inoculum of only 10 to 100 bacteria are required to cause the disease. Epidemics usually occur in areas with crowding and poor sanitary conditions, where transmission from person to person is common or when the organisms contaminate the food or water (Kotloff *et al.*, 1999). Shigellosis is not the most frequent cause of diarrheal disease, but its dysenteric form is the most severe: each year, it kills between 600,000 and 1 million people, mostly children in developing countries.

Shigella is a non-motile, rod shaped, nonspore-forming, lactose fermenting facultative anaerobic

Gram-negative bacterium (Yang *et al.*, 2005; Cheng, 2008). There are 4 species of *Shigella* classified on the basis of biochemical serological differences. Serogroup A: *S. dysenteriae* (12 serotypes), Serogroup B: *S. flexneri* (6 serotypes), Serogroup C: *S. boydii* (23 serotypes) and Serogroup D: *S. sonnei* (1 serotype) (Niyogi, 2005).

Shigellosis is one of the acute enteric disease for which antimicrobial therapy is generally required to manage infection and reduce fecal excretion of the bacterium to prevent further transmission. Although *Shigella* spp. is intrinsically susceptible to all antibiotics that are active against gram-negative bacilli, under antibiotic pressure, they have progressively acquired resistances to commonly recommended drugs (Hirose *et al.*, 2005; Toro *et al.*, 2005). Resistance dissemination among *Shigella* spp. is facilitated by the ability of this genus to acquire mobile genetic elements such as plasmids or transposons (Kotloff *et al.*, 1999).

Shigellosis is the third leading bacterial gastrointestinal diseases in the United States, of which two third are due to *Shigella sonnei* (Cimmons, 2000).

Approximately 900 cases of *S. sonnei*

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0030-9923/2010/0005-0587 \$ 8.00/0

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infection are reported annually in the United Kingdom, and 15 cases of *S. sonnei* infection were reported to the National Disease Surveillance Center in Ireland in 2001.

Similar type of out breaks due indigenous *Shigella sonnei* have been reported from Australia in 1999 (McCall *et al.*, 2000). Taiwan in 2001 to 2003 (Wei *et al.*, 2007), Korea in 1951 and afterwards (Seol, 2006), Bangladesh in 1999-2003 (Talukder *et al.*, 2006).

The scientists have found multiple antibiotic resistance strains of *S. sonnei*. A high proportion of the resistant strains were found to be resistant to some of the most commonly used antibiotics such as tetracycline and streptomycin (Seol, 2006).

In Bangladesh more than 60% of the isolates were resistant to nalidixic acid, 89% to sulfamethoxazole-trimethoprim and 9.5% to ampicillin. In addition, 4% of strains were resistant to multiple antibiotics Amp-Tet-Sxt-St and 4.2% of strains were sensitive to all antibiotics tested. None of the strains were positive for the *set1* gene, whereas 46% were positive for the *sen* gene. Forty-six per cent of the strains (stored at -70°C) harbored the 120 MDa invasive plasmid and representative strains produced keratoconjunctivitis in the guinea pig eye. In addition, three plasmids of approximately 5, 1.8 and 1.4 MDa were found to be present in more than 90% of the strains. A self-transmissible, middle-ranged plasmid (35–80 MDa) carrying the multiple antibiotic resistance genes were found in some strains (Talukder *et al.*, 2006).

In order to ensure appropriate treatment, continual surveillance is required to determine which antibiotics are still active. The people in Azad Kashmir Pakistan face health hazards because of poor sanitation practices *i.e.*, habit of open defecation, lack of hygiene education and use of highly contaminated water. The present research work was aimed at investigating the virulence factors in locally isolated *S. sonnei* and to determine their possible role in infection. The object also was to suggest preventive measures.

MATERIALS AND METHODS

This prospective study was carried out between January 1994 to December 1998 in Azad

Kashmir, which is a mountainous region and located 140 km. north-east of Islamabad (Pakistan). Approximately 4.3 million people live in the state of Azad Kashmir comprising rural and urban populations.

Bacterial strains

Shigella sonnei strains were isolated from stools of patients suffering from diarrhoea admitted at different hospitals of Azad Kashmir (Pakistan), over a 5-year period. The samples were obtained from children (aged 0-5 years) and adults. The study subjects were both male and female. A questionnaire for gathering information including on age, sex, address, patient code number and laboratory result report forms were used to collect data. For the isolation of *Shigella sonnei* a loop full of stool was mixed with 10 ml of sterile buffered peptone water and incubated at 37°C for 24 h. After incubation a loop full of culture was streaked on the SSA and MacConkey agar plates and were incubated at 37°C for 24 h. Non-lactose fermenting colonies (*i.e.* colorless) on MacConkey agar plates were inoculated on XLD agar and incubated at 37°C for 24 h. After incubation, red colonies with 2-4 mm diameter were marked and suspected colonies were subjected to subsequent Gram staining (gram negative short rod). All plates were incubated aerobically at 37°C for 24 hours. From amongst the suspected *Shigella sonnei* from both SSA and MacConkey agar, the non-lactose-fermenting (NLF) colonies were biochemically identified on Urea, Triple Sugar Iron (TSI), Sulphide-Sulphide-indol and motility medium (SIM), and Siminuous Citrate tests. Serotyping was determined by Kligler Iron agar (DIFCO).

Chemicals and media

Chemicals and antibiotics used in this study were obtained from Sigma Chemicals Co. and were of molecular biology grade. The culture media were purchased from DIFCO Laboratories DIFCO (USA). LB medium was used for the cultivation of bacteria and Muller Hinton agar DIFCO was used for susceptibility testing. Antibiotic susceptibility discs used were from OXID, England and also prepared in the cell and molecular biology laboratory. Antibiotics used in these studies were

amikacin (Ak), amoxicillin (Am), ampicillin (A), carbenicillin (Ca), cefixime (Cfm), ceftizoxime (Cxm), ceftriaxone (Cz), cephalothin (Cl), chloramphenicol (C), ciprofloxacin (Cip), cotrimoxazole (Co), enoxacin (E), erythromycin (Er), gentamicin (G), kanamycin (K), nalidixic acid (Na), penicillin (P), streptomycin (S), sulfamethoxazole-trimethoprim (SxT) and tetracycline (T). Stock solutions (10µg/ml) of antibiotics were made in distilled water. Chloramphenicol was dissolved in ethanol. All solutions were sterilized by Millipore (0.45µm) filters and refrigerated.

Antimicrobial sensitivity testing

Antibiotic susceptibility tests of the collected strains of *Shigella sonnei* were performed by antibiotic disc diffusion method (Bauer *et al.*, 1966) using filter paper discs. The minimum inhibitory concentrations (MICs: 25µg/ml, 50µg/ml, 100µg/ml, and 300µg/ml) of fifteen commonly used antibiotics were determined by agar dilution method and the MIC was defined, as the lowest concentration on which there was no visible growth. Reference strains *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were tested regularly as controls according to the National Committee for Clinical Laboratory Standards (NCCLS, 1993).

Plasmid DNA isolation

Plasmid DNA was isolated from the multiple antibiotics resistant strains according to Birnboim and Doly (1979) and was done to separate, identify and purify the plasmid DNA through agarose gel (Meyers *et al.*, 1976). The plasmid DNA was purified by removal of RNA present in the solution. RNA was removed with the help of RNase. To estimate the size of plasmid DNA, DNA Marker (Lambda DNA cut with *Hind*-III) was used. After gel electrophoresis, plasmid DNA was stained with fluorescent, intercalating dye, ethidium bromide. DNA bands were visualized under UV illuminator. Photographs of the gel were positioned over a short-wave UV light source that was taken with the help of gel documentation system GDS-5000 (UVP) and the images of DNA bands were obtained. Individual plasmids of multiplasmid isolates were separated in 1% low-melting agarose gel. Various plasmids

DNA bands were individually cut out of the gel with a sharp razor, extracted, and purified by the usual molecular biological techniques (Weislander, 1979).

Transformation

All the isolates were tested for the ability to transfer their determinants. *E. coli* HB101 (plasmid less and sensitive to antibiotics) were transformed with different individually isolated plasmids. For this, 5 µl of plasmid DNA of Multiple drug resistant (MDR) *Shigella sonnei* was added to competent cells of *E. coli* HB101, prepared, incubated on ice for 30 minutes and then at 42°C for two minutes. One ml of pre-warmed LB broth was then added to this mixture and re-incubated at 37°C at 60 rpm for 80 minutes. The whole mixture was then spread on two different Luria-Bertani agar plates containing ampicillin (100 µg/ml), chloramphenicol (100 µg/ml) sulfamethoxazole-trimethoprim (SxT-100 µg/ml), streptomycin (S-100 µg/ml) and tetracycline (T-100 µg/ml) and incubated at 37°C overnight (Sambrook *et al.*, 1989).

RESULTS AND DISCUSSION

Shigellosis is primarily a childhood disease in both developed and developing countries, whereas epidemic shigellosis affects all age groups including Pakistan (Keusch and Bennish, 1991; Ahmad and Shakoori, 1996). However, the information about the etiology and drug sensitivity pattern of bacterial strains is lacking due to the lack of diagnostic facilities.

In this study, 83 strains of *S. sonnei* were isolated and during the study period, out of 83 *S. sonnei*, in 1994, 19 (8.4%) strains were recovered, where as this number was 26 (15.5%), 12 (6.3%), 10 (8.2%) and 16 (7.8%) in 1995, 1996, 1997 and 1998 respectively. The highest number of *S. sonnei* was recovered in 1995 (15.5%) followed by (8.4%) in 1994, (8.2%) in 1997, (7.8%) in 1998 and the lowest number was recovered in 1996 (6.3%). The highest proportion of stool specimens infected with *S. sonnei* was observed in the age group of >40-50 years (14.3%) followed in >0-5 years (11.1%), >50-60 years (9.0%), >60 years (7.7%), >10-20 years (4.4%), >5-10 years (3.7%) and >20-30 years (3.3). The lowest infestation was observed in the age

groups >30-40 years (2.9%).

Almost similar results were reported by earlier workers Ahmad *et al.* (2003) they observed shigellosis in all age groups, but slightly higher in the age groups of >10-20 and 20-30 years. Khalil *et al.* (1998) reported the highest infestation of *Shigella* in the age groups of 18-23 and 24-35 years. Similarly, Bhattacharya *et al.* (2005) reported that the majority (79%) of *Shigella* species were isolated from children aged less than five years in a recent study in Eastern Nepal.

Antimicrobial sensitivity testing

In the present study, *Shigella sonnei* isolates were resistant to 17 of 20 antibiotics tested.

Overall 65.1% *Shigella sonnei* isolates were resistant to penicillin (P) followed by 53.0% to carbenicillin, (Ca), 51.8% to tetracycline (T), 50.6% to erythromycin (Er), 49.4% to ceftizoxime (CXM), 45.8% to ampicillin (A), 42.2% to amoxicillin (Am), 37.3% to sulfamethoxazole-trimethoprim (SxT), 36.1% to kanamycin (K), 33.7% to amikacin (Ak), 32.5% to co-trimoxazole (Co), 31.3% to streptomycin (S), 25.3% to chloramphenicol (C), 24.1% to nalidixic acid (Na), 21.7% to gentamicin (G), 20.5% to ceftriaxone (Cz) and 19.3% to cephalothin (Cl). All *Shigella sonnei* isolates were sensitive to cefixime (Cfm), ciprofloxacin (CIP) and enoxacin (E).

These results are comparable with the results of a previous study of McCall *et al.* (2000), where they reported that the antibiotic sensitivity testing revealed that all *S. sonnei* isolates were uniformly resistant to ampicillin, amoxicillin-clavulanate and trimethoprim-sulfamethoxazole, and were uniformly sensitive to ciprofloxacin, cefotaxime and gentamicin. Talukder *et al.* (2006) reported in a study in Bangladesh that more than 60% of the isolates were resistant to nalidixic acid, 89% to sulfamethoxazole-trimethoprim and 9.5% to ampicillin. In addition, 4% of strains were resistant to multiple antibiotics Amp-Tet-Sxt-St and 4.2% of strains were sensitive to all antibiotics tested. Similarly, Penatti *et al.* (2007) observed in a study in Southeast Brazil that *S. sonnei* strains were mainly resistant to sulfamethoxazole (100.0%) and tetracycline (96.7%) and, to a lesser extent, to ampicillin (6.7%) and streptomycin (26.7%). But a

contradictory study was presented by Kapperud (1995) where they have reported that, 11 *S. sonnei* isolates were fully susceptible to all 13 antimicrobial agents tested, except that 1 isolate showed ampicillin resistance that was reversed by clavulanate.

The MICs of twenty antibiotics against eighty three of *S. sonnei* are shown in a comparative account of the antibiotics resistance of isolates at four levels 25µg/ml, 50µg/ml, 100µg/ml and 300µg/ml in Table I. Generally, the isolates showed the highest frequency of resistance against penicillin (P) at all the four levels. The lowest frequency of resistance was against ceftriaxone (Cz) at all the four levels of antibiotics screened. At 100µg/ml level the isolates showed a considerable decrease in the resistance frequency of almost all the antibiotics tested.

The resistance of *S. sonnei* to doses as high as 300µg/ml is alarming, because, if *S. sonnei* become resistant to such high levels of antibiotics, the treatment of disease with antibiotics would become quite difficult. Ahmed and Shakoori (1996) reported highest frequency of resistance against septran at 50 and 100µg/ml. Chloramphenicol resistance was 88.8%. In a recent study in Pakistan, Ahmed and Shakoori (2001) documented 50% resistance of *Shigella* strains and Ahmed *et al.* (2003), in Northern Areas of Pakistan reported 14.3% resistance of *Shigella* strains against chloramphenicol.

Multiple drug resistance was observed in this study ranging from three to ten drugs. Out of eighty three isolates, screened for antibiotic resistance, 34% were resistant to three or more antibiotics at 25µg/ml, 29% were resistant to three or more antibiotics at 50µg/ml, 12% were resistant to three or more antibiotics at 100µg/ml and 5% were resistant to three or more antibiotics at 300µg/ml. The resistant isolates showed different patterns of antibiotics resistance. The most common pattern was PCaA at all the four levels shown in Table II.

Analogous results were reported by other investigators in many countries including Pakistan (Ahmed and Shakoori, 2001; Ahmed *et al.*, 2003).

Total 48 strains of *S. sonnei* were processed for isolation of plasmids and only 14 (29.2%) isolates of *S. sonnei* carried plasmids. These were

Table I. Occurrence of antibiotics resistance of 83 *Shigella sonnei* at four different concentrations, isolated from stools of patients with diarrhoea of Azad Kashmir, 1994- 1998.

Antibiotics	No. of resistant isolates at			
	25 µg/ml	50 µg/ml	100 µg/ml	300 µg/ml
Amikacin (Ak)	28(33.7%)	26 (31.3%)	12 (14.4%)	2 (2.4%)
Ampicillin (A)	38(45.8%)	35 (42.2%)	15 (18.1%)	5 (6.0%)
Amoxicillin (Am)	35(42.2%)	32 (38.5%)	14 (116.9%)	4 (4.8%)
Carbenicillin (Ca)	44(53.0%)	42 (50.6%)	19 (22.9%)	9 (10.8%)
Cefixime (Cef)	00(0.0%)	00 (0.0%)	00 (0.0%)	00 (0.0%)
Ceftizoxime (CXM)	41(49.4%)	38(45.8%)	18 (21.7%)	8 (9.6%)
Ceftriaxone (Cz)	17(20.5%)	14 (16.9%)	5 (6.0%)	1 (1.2%)
Cephalothin (Cl)	16(19.3%)	13 (15.7%)	5 (6.0%)	1 (1.2%)
Chloramphenicol (C)	21(25.3%)	19 (22.9%)	7 (8.4%)	2 (2.4%)
Ciprofloxacin (Cip)	00(0.0%)	00(0.0%)	00 (0.0%)	00 (0.0%)
Co-trimoxazole (Co)	27(32.5%)	24 (28.9%)	9 (10.8%)	3 (3.6%)
Enaxacin (E)	00(0.0%)	00 (0.0%)	00 (0.0%)	00 (0.0%)
Erythromycin (Er)	42(50.6%)	37 (44.6%)	16 (19.3%)	6 (7.2%)
Gentamicin (G)	18(21.7%)	16 (19.3%)	6 (7.2%)	2 (2.4%)
Kanamycin (K)	30(36.1%)	27 (32.5%)	10(12.0%)	4 (4.8%)
Nalidixic acid (Na)	20(24.1%)	18 (21.7%)	7 (8.4%)	2 (2.4%)
Penicillin (P)	54(65.1%)	53(63.8%)	23 (27.7%)	10 (12.0%)
Sulfamethoxazole- Trimethoprim (SxT)	31(37.3%)	28 (33.7%)	11 (13.2%)	3 (3.6%)
Streptomycin (S)	26(31.3%)	23 (27.7%)	9 (10.8%)	2 (2.4%)
Tetracycline (T)	43(51.8%)	40 (48.2%)	17(20.5%)	7 (8.4%)

found resistant to three or more antibiotics used in this research work. The number of plasmids varied from one to seven. Although plasmid pattern was determined by the presence or absence of a single plasmid within a group of strains, a number of small plasmids were found to be present universally in all the strains of a particular serotype.

In *S. sonnei*, the analysis of plasmid DNA revealed that all the strains contained a heterogeneous population of plasmids ranging between >23.1 kb to <2.0 kb (Fig. 1, Table III). The molecular size of all plasmids was determined by comparison with a bacteriophage lambda DNA digest with *Hind*-III. The most dominant plasmids were 4.3 Kb, 2.3 Kb, 23.1 Kb, 2.0 Kb, >23.1 Kb, 9.4 Kb and <2.0 Kb. The frequency with which they were encountered was 71.4%, 57.1%, 57.1%, 42.8%, 35.7%, 35.7% and 28.6% respectively. Other plasmids were observed in lesser frequency. The frequency of 6.5 Kb plasmid was 14.3%, and for >4.3 Kb it was 14.3%.

Based on molecular weight, the pattern of different plasmids was also very diverse. Depending

on the number of plasmids, individual strains were grouped into different patterns. Nine different plasmid patterns, designated P1-P9, were found among the 14 strains. Two strains (14.3%) had pattern P1 (5 plasmids), where as two strains (14.3%) had pattern P2 (1 plasmid), while two strains (14.3%) had pattern P3 (5 plasmids), where as another two strains (14.3%) had pattern P4 (3 plasmids), another group of two strains (14.3%) had P5 (3 plasmids), where as one strain (7.1%) had P6 (5 plasmids), while one strain (7.1%) had P7 (5 plasmids), where as another one strain (7.1%) had P8 (2 plasmids) and the remaining one strain (7.1%) had pattern P9 (2 plasmids).

Based on molecular weight, the pattern of different plasmids was also very diverse. Depending on the number of plasmids, individual strains were grouped into nine different plasmid patterns and were found among (MDR) *S. sonnei* strains. The comparable results have been presented in a previous study by Hoe *et al.* (2005). They reported that the heterogeneous plasmid patterns were observed in all *Shigella* spp. while four common

Table II.- Multiple antibiotic resistance patterns occurring in *Shigella sonnei* isolated from stools of patients with diarrhoea of Azad Kashmir, 1994- 1998.

Antibiotics resistance patterns	Percent of resistant isolates at ($\mu\text{g/ml}$)			
	25	50	100	300
P, Ca, A	34	29	12	5
P, A, T	31	24	10	4
P, Ca, A, T	26	19	7	3
P, A, T, Er	20	15	7	2
P, Ca, A, Er	17	11	5	2
P, Ca, A, T, Er	14	9	4	2
P, C, A, T, CXM	12	8	3	1
P, Ca, T, CXM, K	9	6	2	1
P, Ca, A, Er, K, Co	7	4	2	1
P, A, T, Er, K, Co	4	2	1	1
P, Ca, A, T, Er, Co, SxT, Am	3	1	1	-
P, A, C, Er, K, Co, Am, Ak, S, Na	2	1	-	-
P, Ca, A, T, Co, Am, Ak, S, Na, G	1	-	-	-
P, Ca, A, K, Am, Na, G, C, Cl, Cz	1	-	-	-

Key: A, Ampicillin; AK, Amikacin; Am, Amoxicillin; Ca, Carbenicillin; Cef, Cefixime; CXM, Ceftizoxime; CZ, Ceftriaxone; Cl, Cephalothin; C, Chloramphenicol; Co, Cotrimoxazole; Er, Erythromycin; G, gentamicin; K, Kanamycin; Na, Nalidixic acid; P, Penicillin; SxT, Sulfamethoxazole-Trimethoprim; S, Streptomycin; T, Tetracycline.

small plasmids were found in *S. sonnei* isolates. The 2.10 kb plasmid was only seen in *S. sonnei* and multi-drug resistance in *S. sonnei* may be associated with the 14.8 kb plasmids. The results of our study are also comparable with the results of Farshed *et al.* (2006) where they observed in a recent study in Iran, that all the *Shigella* spp. isolates harbored multiple plasmids, with an average of 9.5 plasmids (range, 5 to 14 plasmids) in each isolate of all strains and a mean of 10 plasmids in each isolate of *S. sonnei*. The sizes of the plasmids from among all isolates ranged from 1 to 21 kb. Similarly, Dutta *et al.* (2002) reported in a previous study, that the plasmid profiles of *S. flexneri* 2a and *Sonnei* strains indicated presence of large plasmid (approx. 210 kb) and multiple copies (4-6 copies) of smaller plasmids in almost all strains. Recently, Talukder *et al.* (2006) reported in a study in Bangladesh that forty-six per cent of the strains (stored at -70°C) harbored the 120 MDa invasive plasmid and representative strains produced keratoconjunctivitis in the guinea pig eye. In addition, three plasmids of approximately 5, 1.8 and 1.4 MDa were found to be

present in more than 90% of the strains.

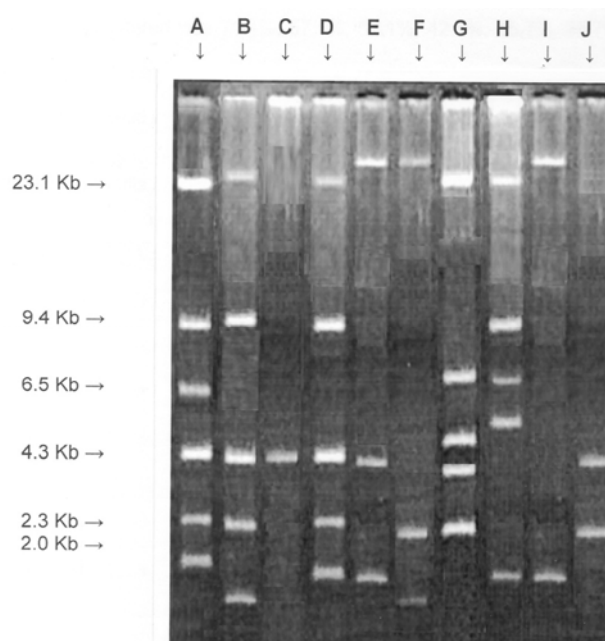


Fig. 1. Plasmid profile (P1-P9) of representative *S. sonnei* strains isolated from fecal samples of patients with gastroenteritis in Azad Kashmir. (Lane A = marker λ DNA cut with *Hind*-III; Lane B, BSs-810; Lane C, BSs-812; Lane D, BSs-819; Lane E, BSs-832; Lane F, BSs-845; Lane G, BSs-850; Lane H, BSs-8009; Lane I, BSs-8015 and Lane J, BSs-8030).

Transfer of antimicrobial resistance determinants and antimicrobial sensitivity testing

Of the 14 *S. sonnei* strains, the plasmids of 13 strains were processed for transformation into *E. coli* HB101 separately for ampicillin (MIC-100 $\mu\text{g/ml}$), chloramphenicol (MIC-100 $\mu\text{g/ml}$) and sulfamethoxazole-trimethoprim (MIC-100 $\mu\text{g/ml}$), plasmids of 8 strains (61.5%) for only ampicillin, 7 (53.8%) for chloramphenicol, and 6 (46.1%) for sulfamethoxazole-trimethoprim resistance. Of the 13 transformations, 11 (84.6%) were successfully accomplished as *E. coli* HB101 acquired antibiotic resistance to ampicillin, chloramphenicol and sulfamethoxazole-trimethoprim. Plasmids of three strains (no. BSs-812, BSs-845 and BSs-8015) were successfully transferred to *E. coli* Hb101 shown by the acquisition of resistance to ampicillin, and plasmids of another three strains (no. BSs-819, BSs-850 and BSs-8030) with chloramphenicol resistance

Table III.- Plasmid profile analysis of *S. sonnei* total no. of strains (n=14).

No. of strains	Presence of plasmid with Molecular weight (Kb) of:									Plasmid pattern
	>23.1	23.1	9.4	6.5	>4.3	4.3	2.3	2.0	<2.0	
2	-	+	+	-	-	+	+	-	+	P1
2	-	-	-	-	-	+	-	-	-	P2
2	-	+	+	-	-	+	+	+	-	P3
2	+	-	-	-	-	+	-	+	-	P4
2	+	-	-	-	-	-	+	-	+	P5
1	-	+	-	+	+	+	+	-	-	P6
1	-	+	+	+	+	-	-	+	-	P7
1	+	-	-	-	-	-	-	+	-	P8
1	-	-	-	-	-	+	+	-	-	P9

Table IV.- Transformation of plasmids of *S. sonnei* in to *E. coli* Hb101.

Sample No.	No. of plasmids	Molecular weight of plasmids which were individually transferred to <i>E. coli</i> HB101.	Transformed plasmids that conferred antibiotic resistance.
810	5	23.1Kb,9.4Kb,4.3Kb,2.3Kb,<2.0Kb.	23.1Kb
832	3	>23.1Kb,4.3Kb,2.0Kb.	>23.1Kb,4.3Kb,2.0Kb.
8009	5	23.1Kb,9.4Kb,6.5Kb,>4.3Kb,2.0Kb.	23.1Kb

were also successfully introduced into *E. coli* HB101. Plasmids of 8 strains resistant to ampicillin, 7 strains resistant to chloramphenicol, and 6 strains resistant to sulfamethoxazole-trimethoprim were also successfully introduced into *E. coli* HB101.

In some multiple plasmid strains (no. BSs-810, BSs-832 and BSs-8009), all the DNA bands of different molecular sizes were cut out of the gel, extracted, purified and then successfully transferred to *E. coli* HB101 individually. The plasmids (>23.1 and 23.1 Kb) could only confer ampicillin, chloramphenicol and sulfamethoxazole-trimethoprim resistance to the competent cells of *E. coli* HB101 (Table IV).

These results are comparable with the results of a previous study by Talukder *et al.* (2006) where they reported that a self-transmissible, middle-ranged plasmid (35–80 MDa) carrying the multiple antibiotic resistance genes were found in some strains.

Conjugative plasmids encoding resistance to antibiotics have been detected in numerous studies on *S. sonnei* (DeLappe *et al.*, 2003). The experiment in the present study demonstrated that middle-range plasmids were self-transmissible, conferring

resistance to ampicillin, tetracycline and trimethoprim-sulfamethoxazole. The co-transfer of streptomycin resistance was not observed, suggesting that the resistance determinants of this antibiotic in *S. sonnei* are not associated with the conjugative plasmids. In contrast to the study of Vargas *et al.* (1999), which showed that 100% of *S. sonnei* strains isolated between 1996 and 1998 were positive for ShET-2 (sen), Talukder *et al.* (2006) found that only 46% of *S. sonnei* strains were positive for this gene. In addition, a correlation between the presence of the (120 MDa) plasmid and the sen gene was observed. Although *S. sonnei* is a more common problem in the developed and industrialized countries, this study underlines a significant burden of this pathogen in overall *Shigella* infections in Pakistan, with strains of heterogeneous traits.

CONCLUSION

In conclusion, there is a significant increase in resistance to several commonly-used antimicrobial agents.

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(Received 20 June 2009, revised 5 January 2010)