Pakistan J. Zool., vol. 41(6), pp. 495-503, 2009.

Antimicrobial Resistance and Plasmid Profile Analysis of Clinically Isolated *Shigella dysenteriae* in Azad Kashmir, Pakistan*

Basharat Ahmed, Farah. R. Shakoori, S. S. Ali and A. R. Shakoori

Department of Zoology, University of the Punjab, New Campus, Lahore (BA, SSA), Department of Zoology, Government College University, Lahore (FRS) and School of Biological Sciences, University of the Punjab, Lahore, Pakistan (ARS)

Abstract.- The antimicrobial susceptibility patterns for 134 *S. dysenteriae* 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 followed by carbenicillin, ampicillin, tetracycline, erythromycin, ceftizoxime, kanamycin, co-trimoxazole, piperacillin, amoxicillin, amikacin, streptomycin, nalidixic acid, gentamicin, chloramphenicol, cephalothin and ceftriaxone. All *S. dysenteriae* 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 most common antibiotics resistance pattern was PCaA. The plasmids were observed in 29.9% MDR strains of *S. dysenteriae* which were found resistant to three or more antibiotics. The number of plasmids varied from one to seven. 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. dysenteriae*, antibiotic resistance, R-plasmid, Azad Kashmir, Pakistan.

INTRODUCTION

Shigella causes bacillary dysentery, which remains a significant threat to public health. It is a rod-shaped non-motile, nonspore-forming, facultative anaerobic Gram-negative and lactose-fermenting bacterium (Yang *et al.,* 2005). *Shigella* spp. are 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 Sergoroup D: *S. sonnei* (1 serotype) (Niyogi, 2005).

Shigella dysenteriae serotype 1, which possesses the cytotoxic Shiga toxin (Stx), causes deadly epidemics in many of the poorer countries (Sansonetti, 2001). Each of the *Shigella* genomes includes a virulence plasmid that encodes conserved primary virulence determinants mostly acquired via bacteriophage-mediated lateral gene transfer. The *Shigella* spp. has hence become highly specific human pathogen with variable epidemiological and pathological features (Yang *et al.*, 2005).

The most severe forms, encountered with *S. dysenteriae* type 1 (the "Shiga bacillus") in children under 5 years of age, lead to a mortality rate of 10 to 30% during outbreaks. *Shigella* spp. can be transmitted by contaminated food and water and through person-to-person contact. In developing countries with unsafe water supplies and substandard hygiene, shigellosis is widespread and causes extensive outbreaks. In industrialized countries, this disease has become rare. The diarrhea is often bloody. Shigellosis usually resolves in 5 to 7 days.

Shigellosis can usually be treated with antibiotics. The antibiotics commonly used for treatment are ampicillin, trimethoprim/ sulfamethoxazole, nalidixic acid, or ciprofloxacin. Appropriate treatment kills the *Shigella,* but unfortunately, indiscriminate use of the drugs and horizontal gene transfer has led to *Shigella* species becoming resistant to commonly used antibiotics (Noriega *et al.,* 1999).

At least three periods of epidemic outbreaks of dysentery due to *S. dysenteriae* 1 have been recorded previously in the Indian subcontinent, in

* Part of Ph.D. thesis of first author.

** Corresponding authors: Email: arshak@brain.net.pk

0030-9923/2009/0006-0495 $ 8.00/0

1972-1973, 1983-1984, and 1993-1994. Despite improvement of municipal water supplies and sanitation, shigellosis still occurs frequently. This raises important questions about the causes of shigellosis, its transmission, epidemiology, and the effectiveness of public health measures in overcoming this illness. Indiscriminate use of antibiotics in this region has resulted in the *Shigella* strains becoming resistant to multiple antibiotics. Most of the *Shigella* strains isolated from patients are resistant to ampicillin (AMP), sulfamethoxazole-trimethoprim (SXT), and nalidixic acid (Hossain *et al.,* 1998).

In order to ensure appropriate treatment, continual surveillance is required to determine the efficacy of antibiotics in use. 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. dysenteriae* and their possible role in infection. The object was also to suggest preventive measures. The strains of *S. dysenteriae* resistant to commonly used antibiotics have also been screened for plasmid DNA. The isolated plasmids have been characterized. The plasmid DNA of multiple drug resistant (MDR) *S. dysenteriae* isolates will be transformed into plasmid-less *E. coli* HB101 strains.

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 dysenteriae strains were isolated from stools of patients suffering from diarrhea 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 *S. dysenteriae* 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 37ºC for 24 hours. From amongst the suspected *S. dysenteriae* 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 Siminous 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), co-trimoxazole (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 (0.45mµ) filters and refrigerated.

Antimicrobial sensitivity testing

Antibiotic susceptibility tests of the collected strains of *S. dysenteriae* were performed by antibiotic disc diffusion method (Bauer *et al*., 1966) using filter paper discs. The minimum inhibitory concentrations of fifteen commonly used antibiotics were determined by agar dilution method. Reference strains *Escherichia coli* ATCC 25922 and *Psdeudomonas 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). It was treated with RNase. To estimate the size of plasmid DNA, Lambda DNA cut with *Hind*-III was used as marker. The images of DNA bands were obtained on the gel documentation system GDS-5000 (UVP). 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

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) *S. dysenteriae* was added to competent cells of *E. coli* HB101, prepared, incubated on ice for 30 minutes and then at 42ºC for two minutes. One ml of pre-warmed LB broth was then added to this mixture and re-incubated at 37ºC at 60 rpm for 80 minutes. The whole mixture was then spread on two different LB 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 37ºC overnight (Sambrook *et al.,* 1989).

RESULTS

In this study during the five year study period, 134 strains of *S. dysenteriae* were isolated and 30 (13.3%) strains were recovered in 1994, where as this number was 28 (16.7%), 32 (16.8%), 17 (13.9%) and 27 (13.1%) in 1995, 1996, 1997 and 1998, respectively. The highest number of *S. dysenteriae* was recovered in 1996 (16.8%) followed by 16.7% in 1995, 13.9% in 1997, 13.3% in 1994 and 13.1% in 1998.

The highest proportion of stool specimens infected with *S. dysenteriae* was in the age group >30-40 years (32.3%) followed by >40-50 years (28.6%), >60 years (23.0%), >50-60 years (18.2%), >5-10 years (16.0%), >10-20 years (14.7%) and >20-30 years (13.2%). The lowest infestation was observed in the age group >0-5 years (12.9%).

Antimicrobial sensitivity testing

Overall 64.9% *S. dysenteriae* isolates were resistant to penicillin (P), followed by 54.5% to carbenicillin, (Ca), 52.9% to tetracycline (T), 51.5% to ampicillin (A), 50.7% to erythromycin (Er), 46.3% to ceftizoxime (CXM), 43.3% to co-trimoxazole (Co), 40.3% to kanamycin (K), 38.8% to sulfamethoxazole-trimethoprim (SxT), 35.8% to amikacin (Ak), 32.8% to amoxicillin (Am), 30.6% to nalidixic acid (Na), 29.8% to streptomycin (S), 29.1% to gentamicin (G), 25.4% to chloramphenicol (C), 21.6% to cephalothin (Cl), and 18.6% to ceftriaxone (Cz). All *S. dysenteriae* isolates were sensitive to cefixime (Cfm), ciprofloxacin (CIP) and enoxacin (E) (Table I).

The MICs of twenty antibiotics against 134 strains of *S. dysenteriae* are shown in Table I. Generally, the isolates showed the highest frequency of resistance against penicillin (P) at all the concentrations. The lowest frequency of resistance was against ceftriaxone (CZ). At 100µg/ml concentration the isolates showed considerable decrease in the resistance frequency of almost all the antibiotics tested. Multiple drug resistance was observed in this study ranging from three to ten drugs. Out of 134 isolates, 55% were resistant to three or more antibiotics at 25µg/ml, 50% were resistant to three or more antibiotics at 50µg/ml, 16% were resistant to three or more antibiotics at 100µg/ml and 8% 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 (Table II).

Table I.- Occurrence of resistance against four different concentrations of antibiotics in 134 isolates of *S. dysenteriae*.

Antibiotics	No. of resistant isolates at			
	25 µg/ml	50 µg/ml	100 µg/ml	300 µg/ml
Amikacin (Ak)	48(35.8%)	46(34.3%)	21 (15.7%)	5 (3.7%)
Ampicillin (A)	69(51.5%)	66 (49.2%)	28 (20.8%)	18 (13.4%)
Amoxicillin (Am)	44(32.8%)	41 (30.6%)	17 (12.7%)	4 (2.9%)
Carbenicillin (Ca)	73(54.5%)	70 (52.2%)	43 (32.1%)	19 (14.2%)
Cefixime (Cef)	00(0.0%)	00 (0.0%)	00 (0.0%)	00 (0.0%)
Ceftizoxime (CXM)	62(46.3%)	58 (43.3%)	33(24.6%)	13 (9.7%)
Ceftriaxone (Cz)	25(18.6%)	21 (15.7%)	9 (6.7%)	2 (1.5%)
Cephalothin (Cl)	29(21.6%)	26 (19.4%)	10 (7.5%)	3 (2.2%)
Chloramphenicol (C)	34(25.4%)	31(23.1%)	12 (8.9%)	4 (2.9%)
Ciprofloxacin (Cip)	00(0.0%)	00(0.0%)	00 (0.0%)	00 (0.0%)
Co-trimoxazole (Co)	58(43.3%)	54 (40.3%)	27(20.1%)	6 (4.5%)
Enaxacin (E)	00(0.0%)	00 (0.0%)	00 (0.0%)	00 (0.0%)
Erythromycin (Er)	68(50.7%)	61(45.5%)	32(23.9%)	14(10.4%)
Gentamicin (G)	39(29.1%)	33 (24.6%)	11 (8.2%)	5 (3.7%)
Kanamycin (K)	54(40.3%)	52 (38.8%)	39(29.1%)	11 (8.2%)
Nalidixic acid (Na)	41(30.6%)	36 (26.9%)	13 (9.7%)	4 (2.9%)
Penicillin (P)	87(64.9%)	82 (61.2%)	61(45.5%)	27(20.1%)
Sulfamethoxazole-Trimethoprim (SxT)	52(38.8%)	48 (35.8%)	22(16.4%)	7 (5.2%)
Streptomycin (S)	40(29.8%)	37(27.6%)	15(11.2%)	10 (7.5%)
Tetracycline (T)	71(52.9%)	67(50.0%)	38(28.3%)	15(11.2%)

Table II.- Multiple antibiotic resistance patterns occurring in *S. dysenteriae* isolated from various clinical sources of Azad Kashmir, 1994- 1998.

Antibiotics resistance patterns*	% of resistant isolates at (µg/ml)			
	25	50	100	300
P, Ca, A	55	50	16	8
P, Ca, T	50	44	12	5
P, Ca, A, T	46	41	10	4
P, A, T, Er	42	37	9	3
P, Ca, A, Er	35	31	8	3
P, Ca, A, CXM	34	29	7	2
P, Ca, A, T, Er	29	24	5	2
P, C, A, T, CXM	27	22	4	1
P, Ca, T, CXM, K	20	17	3	1
P, Ca, A, T, K, Co	13	12	3	1
P, Ca, A, T, Er, Co, SxT, Am	10	9	2	-
P, C, A, K, Co, SxT , Am, Ak	8	7	1	-
P, A, C, Er, K, Co, Am, Ak, S, Na	2	2	-	-
P, Ca, A, T, Co, Am, Ak, S, Na, G	1	1	-	-
P, Ca, A, K, Am, Na, G, C, Cl, Cz	1	1	-	-

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

A total of 134 strains of *S. dysenteriae* were processed for isolation of plasmids and only 43 (29.9%) strains carried plasmids. These strains were found resistant to three or more antibiotics used. 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. The analysis of plasmid DNA of *S. dysenteriae* 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 most dominant plasmids were 23.1, 4.3, 6.5, 2.3, <4.3, 2.0 and 9.4 Kb. The frequency with which they were encountered was 100%, 81.4%, 55.8%, 48.8%, 41.9%, 34.9% and 27.9% respectively. Other plasmids were observed in lesser frequency. The frequency of <23.1 Kb plasmid was 25.6%, for <2.0 Kb it was 23.2% for <6.5 Kb it was 2.3% and for >2.3 Kb it was also 2.3%.

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

Table III.- Plasmid profile analysis of *S. dysenteriae* total no. of strains (n=43).

No. of strains	Presence of plasmid with molecular weight (Kb) of											Plasmid
	23.1	<23.1	9.4	6.5	<6.5	4.3	<4.3	>2.3	2.3	2.0	<2.0	pattern
11	+	-	-	+	-	+	+	+	-	-	-	P1
7	+	+	-	+	-	+	+	-	+	+	-	P2
6	+	-	+	-	-	-	-	-	+	+	-	P3
5	+	-	-	+	-	+	-	-	+	-	+	P4
4	+	-	+	-	-	+	-	-	-	-	-	P5
4	+	+	-	-	-	+	-	-	-	-	+	P6
3	+	-	-	-	-	+	-	-	+	-	-	P7
2	+	-	+	-	-	-	-	-	-	+	-	P8
1	+	-	-	+	+	+	-	-	-	-	+	P9

Table IV.- Antibiotic resistance of *E. coli* HB101 transformed with different plasmids of *S. dysenteriae* isolates.

Sample no.	No. of plasmids	Molecular weight of plasmids which were individually transferred to *E. coli* HB101	Transformed plasmids that conferred antibiotic resistance
346	4	23.1Kb, 6.5Kb, 4.3Kb, 2.3Kb, <2.0Kb.	23.1Kb.
378	3	23.1Kb, <23.1Kb, 4.3Kb, <2.0Kb.	23.1Kb,<23.1Kb.
3025	2	23.1Kb, 9.4Kb, 2.0Kb.	23.1Kb.

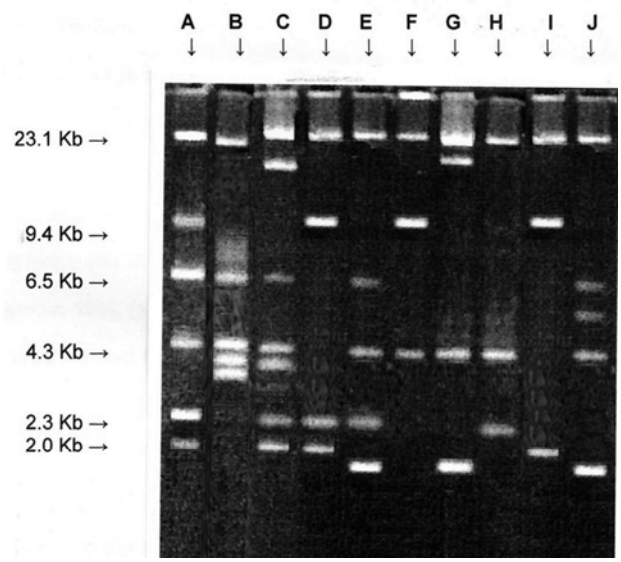

Fig. 1. Plasmid profile (P1-P9) of representative *S. dysenteriae* strains isolated from fecal samples of patients with gastroenteritis in Azad Kashmir. Lane A, marker λ DNA cut with Hind III; Lane B, BSd-304; Lane C, BSd-307; Lane D, BSd-315; Lane E, BSd-346; Lane F, BSd-357; Lane G, BSd-378; Lane H, BSd-391; Lane I, BSd-3025 and Lane J, BSd-3031.

plasmid patterns, designated P1-P9, were found among the 43 strains. Eleven strains (25.6%) had pattern P1 (5 plasmids), while seven strains (16.3%) had pattern P2 (7 plasmids), where as six strains (13.9%) had pattern P3 (4 plasmids), while five strains (11.6%) had pattern P4 (5 plasmids), where as four strains (9.3%) had P5 (3 plasmids), while another four strains (9.3%)had P6 (4 plasmids), three strains (6.9%) had P7 (3 plasmids), two strains (4.6%) had P8 (3 plasmids) the remaining one strain (2.3%) had pattern P9 (5 plasmids).

Transfer of antimicrobial resistance determinants and antimicrobial sensitivity testing

Of 43 *S. dysenteriae* strains, the plasmids of 30 strains were processed for transformation of *E. coli* HB101 separately for ampicillin (MIC-100 µg/ml), chloramphenicol (MIC-100 µg/ml) and sulfamethoxazole-trimethoprim (MIC-100 µg/ml), plasmids of 20 strains (66.6%) for only ampicillin, 16 (53.3%) for chloramphenicol, and 14 (46.6%) for sulfamethoxazole-trimethoprim resistance. Of the 30 transformations, 24 (80.0%) were successfully accomplished as *E. coli* HB101 acquired antibiotic resistance to ampicillin, chloramphenicol and sulfamethoxazole-trimethoprim. Plasmids of three

strains (no. BSd-307, BSd-357 and BSd-391) were successfully transferred to *E. coli* Hb101 shown by the acquisition of resistance to ampicillin, and plasmids of another three strains (no. BSd-304, BSd-315 and BSd-3031) with chloramphenicol resistance were also successfully introduced into *E. coli* HB101. Plasmids of 20 strains resistant to ampicillin, 16 strains resistant to chloramphenicol, and 14 strains resistant to sulfamethoxazole-trimethoprim were also successfully introduced into *E. coli* HB101.

In some multiple plasmid strains (no. BSd-346, BSd-378 and BSd-3025), 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 Kb and <23.1 Kb) could only confer ampicillin, chloramphenicol and sulfamethoxazole-trimethoprim resistance to the competent cells of *E. coli* HB101 (Table IV).

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, the highest number of *S. dysenteriae* was recovered in 1996, (16.8%) followed by (16.7%) in 1995, (13.9%) in 1997, (13.3%) in 1994 and the lowest number was recovered in 1998, (13.1%). The highest proportion of stool specimens infected with *S. dysenteriae* was in the age group of >30-40 years (32.3%). The lowest infestation was observed in the age group >0-5 years (12.9%). 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. Oo and Thida (1995) reported the highest infestation of *S. dysenteriae* in the age group of 20-30 years in Yangon, Myanmar. Recently, 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.

Shigellosis is a highly contagious disease caused by *Shigella* spp. and humans are the principal reservoir of infection. Overuse of antibiotics also contributes to antibiotic resistance.

In the present study, 64.9% *S. dysenteriae* isolates were resistant to penicillin followed by 54.5% to carbenicillin, 52.9% to tetracycline, 51.5% to ampicillin, 50.7% to erythromycin, 46.3% to ceftizoxime, 43.3% to co-trimoxazole, 40.3% to kanamycin, 38.8% to sulfamethoxazole-trimethoprim, 35.8% to amikacin, 32.8% to amoxicillin, 30.6% to nalidixic acid, 29.8% to streptomycin, 29.1% to gentamicin, 25.4% to chloramphenicol, 21.6% to cephalothin, and 18.6% to ceftriaxone. All *S. dysenteriae* isolates were sensitive to cefixime, ciprofloxacin and enoxacin. Almost analogous results were documented by Dutta *et al.* (2003), the commonest antimicrobial resistance profile, observed in 97% strains, was resistance to seven antimicrobials: ampicillin, tetracycline, nalidixic acid, amoxicillin, co-trimoxazole, ciprofloxacin, and norfloxacin and in Bangladesh Talukder *et al.* (2003) reported more than 98% of *Shigella dysenteriae* type 1 strains isolated between 1999 and 2001 were resistant to ampicillin, sulfamethoxazole-trimethoprim, and nalidixic acid. In addition similar resistance patterns were reported by Ahmed *et al.* (2000), where *Shigella dysenteriae* type 1 showed high resistance rates (percentage of isolates showing antibiotic resistance) against the commonly used antimicrobial agents: ampicillin, amoxicillin, chloramphenicol, tetracycline, co-trimoxazole, nalidixic acid, sulfonamide, and neomycin, and were completely sensitive to ciprofloxacin. According to Bennis *et al*. (1992) at the International Center for Diarrheal Disease Research in Bangladesh, 71.5% of isolates of *S. dysenteriae* type 1 were found to be resistant to ampicillin, 65.5% to co-trimoxazole, and 57.9% to nalidixic acid. Similar studies in other countries in east Africa have shown similar patterns of resistance. In a diarrhea outbreak in northern Kenya, *Shigella dysenteriae* and *Shigella flexneri* were isolated that were sensitive only to nalidixic acid and furazolidine (Materu *et al.*, 1997).

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. dysenteriae* to doses as high as 300µg/ml is alarming, because, if *S. dysenteriae* 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 one hundred and thirty four isolates, screened for antibiotic resistance, 55% were resistant to three or more antibiotics at 25µg/ml, 50% were resistant to three or more antibiotics at 50µg/ml, 16% were resistant to three or more antibiotics at 100µg/ml and 8% 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. Analogous results were reported by other investigators in many countries including Pakistan (Ahmed and Shakoori, 2001; Ahmed *et al.,* 2003).

Shigella species usually harbor a heterogeneous population of plasmids ranging in number from 2 to as many as 10 (Surdeanu *et al.,* 2000). In the current study analysis of plasmid DNA of *S. dysenteriae* revealed that all the strains contained a heterogeneous population of plasmids ranging between 23.1 kb to <2.0 kb. Almost analogous results were reported in a previous study, Talukder *et al.* (2003) where the analysis of the plasmid DNA of *S. dysenteriae* strains showed that all strains contained multiple plasmids ranging between 140 and 0.8 MDa. Plasmid patterns of different serotypes were found to be different. Within a single serotype multiple patterns were observed, indicating their clonal heterogeneity. 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. dysenteriae* strains. Recently, Hoe *et al.* (2005) reported that all *S. dysenteriae* type 2 isolates harbored the 9.00 kb plasmid.

Haider *et al.* (1988) have reported numerous plasmid patterns in *S. dysenteriae* type 1 isolated from scattered as well as defined geographical origins. They found a number of core plasmids associated with the specificity of individual serotypes irrespective of drug resistance patterns (Talukder *et al.,* 2003). Dutta *et al.* (2003) also reported that the plasmid profile analysis of *S. dysenteriae* type 1 revealed four different profiles (types I to IV), of which the type I profile was the most predominant and was found in 88% strains. All strains had a heavy plasmid of 210 kb. Other types (types II to IV) showed gain or loss of two or three smaller plasmids. Multiple copies (4-6 copies) of smaller plasmids are present in almost all strains. Each of the serotypes presented with unique plasmid profile, hence this can be used as an epidemiological marker tool for strain differentiation.

In this study, the plasmids of (MDR) *S. dysenteriae* strains, were used for transformation of *Escherichia coli* HB101. The 23.1 Kb and <23.1 Kb plasmids could only confer ampicillin, chloramphenicol and sulfamethoxazole-trimethoprim resistance to the competent cells of *Escherichia coli* HB101. These results are comparable with those of Gebre-Yohannes and Drasar (1988) where they reported that the 96% of *S. dysenteriae* type 1 (Shiga's bacillus) strains, harbored conjugative plasmids coding for ACSSuT resistance. After 1980, however, about 50% of isolates of Shiga's bacillus with this resistance (R)-type carried conjugative plasmids that transferred at high frequencies (10 degrees-10(-2)) and that expressed the ACT determinant only. Similarly, Bratoeva and John, Jr. (1994) in a study in Bulgaria reported that the strains of *S. dysenteriae* type 1 were resistant against the trimethoprim/ sulfamethoxazole and these multiresistant strains had contained a 48-kb plasmid that conferred the resistance to a susceptible *E. coli*. Talukder *et al.*

(2003) found the prevalence of type 4 strains increasing along with resistance to multiple antibiotics including nalidixic acid.

REFERENCES

AHMED, B. AND SHAKOORI, A. R., 1996. Multiple antibiotic resistance among *Shigella* species, isolated from patients of shigellosis in Pakistan. *Punjab Univ. J. Zool.,* **11:** 83-88.

AHMED, A.A., OSMAN, H., MANSOUR, A.M., MUSA, H. A., AHMED, A.B., KARRAR, Z. AND HASSAN, H.S., 2000. Antimicrobial agent resistance in bacterial isolates from patients with diarrhea and urinary tract infection in the Sudan. *Am. J. trop. Med. Hyg.,* **63:** 259–263.

AHMED, B. AND SHAKOORI, A.R., 2001. Isolation and characterization of R-plasmid from antibiotic resistant clinical, isolates of *Shigella* species. *Pakistan J. Zool*., **33:** 99-104.

AHMED, K., SHAKOORI, F.R. AND SHAKOORI, A.R., 2003. Aetiology of Shigellosis in northern Pakistan. *J. Hlth. Popul. Nutr.,* **21:** 32-39.

BAUER, A.W., KIRBY, W.M., SHERRTES, J.C. AND TURCK, M., 1966. Antibiotic susceptibility testing by a standard single disk method. *Am. J. clin. Pathol.,* **5:** 493-496.

BENNIS, M.L., SALAM, M.A., HOSSAIN, M.A., MYAUX, J., KHAN, E.H., CHAKRABORTY, J., HENRY, F. AND RONSMANS, C., 1992. Antimicrobial resistance of *Shigella* isolates in Bangladesh, 1983-1990: increasing frequency of strains multiply resistant to ampicillin, trimethoprim-sulfamethoxazole, and nalidixic acid. *Clin. Infect. Dis.,* **14:**1055-1060.

BHATTACHARYA, S., KHANAL, B., BHATTARAI, N.R. AND DAS, M.L., 2005. Prevalence of *Shigella* species and their antimicrobial resistance patterns in Eastern Nepal. *J. Hlth. Popul. Nutr.,* **23:** 339-342.

BIRNBOIM, H.C. AND DOLY, J., 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucl. Acids Res.,* **7:** 1513-1523.

BRATOEVA, M.P. AND JOHN, JR. J.F., 1994. *In vivo* R-plasmid transfer in a patient with a mixed infection of *Shigella* dysentery. *Epidemiol. Infect*., **112:** 247-252.

DUTTA, S., GHOSH, A., GHOSH, K., DUTTA, D., BHATTACHARYA, S.K., NAIR, G.B. AND YOSHIDA, S-I., 2003. Newly emerged multiple-antibiotic-resistant *Shigella dysenteriae* Type 1 strains in and around Kolkata, India, are clonal. *J. clin. Microbiol*., **41:** 5833-5834.

GEBRE-YOHANNES, A. AND DRASAR, B.S., 1988. Transferable or mobilisable antibiotic resistance in *Shigella dysenteriae* types 1, 2, 3, 4, 6 and 7 isolated in Ethiopia during 1974-85. *J. med. Microbiol*., **27:** 285-289.

HAIDER, K., BRADFORD, A.K., TALUKDER, K.A. AND HUQ, M.I., 1988. Plasmid analysis of *Shigella dysenteriae* type 1 isolates obtained from widely scattered geographical locations. *J. clin. Microbiol.,* **26:** 2083-2086.

HOE, C.H., YASSIN, R.M., KOH, Y.T. AND THONG, K.L., 2005. Survey of plasmids profiles of *Shigella* species isolated in Malaysia during 1994-2000. *World J. Microbiol. Biotechnol*., **21:** 271-278.

HOSSAIN, M.A., RAHMAN, M., AHMED, Q.S., MALEK, M. A., SACK, R.B. AND ALBERT, M.J., 1998. Increasing frequency of mecillinam-resistant *Shigella* isolates in urban Dhaka and rural Matlab, Bangladesh: a 6-year observation. *J. Antimicrob. Chemother.,* **42:** 99-102.

KEUSCH, G.T. AND BENNISH, M.L**.,** 1991. Shigellosis. In: *Bacterial infections of humans: epidemiology and control* (eds. A.S. Evans and P.S. Brachman). 2d ed. Plenum, New York. pp. 593-620.

KHALIL, K., KHAN, S. R., MAZHAR, K., KAIJSER, B. AND LINDBLOM, G-B., 1998. Occurrence and susceptibility to antibiotics of *Shigella* species in stools of hospitalized children with bloody diarrhea in Pakistan. *Am. J. trop. Med. Hyg*., **58:** 800–803.

MATERU, S.F., LEMA, O.E., MUKUNZA, H.M., ADHIAMBO, C.G. AND CARTER, J.Y., 1997. Antibiotics resistance of *Vibrio cholerae* and *Shigella* causing diarrhoea outbreaks in the eastern Africa region. *East Afr. med. J.,* **74:** 193–197.

NATIONAL COMMITTEE FOR CLINICAL LABORATORY STANDARDS, 1993. Performance standards for antimicrobial disk susceptibility tests. Document M2-A4, 4th ed., vol. 10, no. 7. National Committee for Clinical Laboratory Standards, Villanova, Pa.

NIYOGI, S. K., 2005. "Shigellosis". *J. Microbiol.,* **43:** 133-143.

NORIEGA, F. R., LIAO, F. M., MANEVAL, D. R., REN, S., FORMAL, S.B. AND LEVINE, M. M., 1999. Strategy for cross-protection among *Shigella flexneri* serotypes. *Infect. Immun*., **67:**782-788.

OO, K. N. AND THIDA, M., 1995. Serotype distribution and antimicrobial susceptibility of *Shigellae* isolated from diarrhoeal patients in Yangon, Myanmar. *J. Diarrh. Dis. Res.,* **13:** 180-182.

SAMBROOK, J., FRITSCH, E. F. AND MANIATIS, T., 1989. *Molecular cloning–a laboratory manual*. Cold Spring Harbor Laboratory, New York. Book **1:** 1.3-1.110.

SANSONETTI, P. J., 2001 Microbes and microbial toxins: paradigms for microbial–mucosal interactions III. Shigellosis: from symptoms to molecular pathogenesis. *Am. J. Physiol. Gastrointest. Liver Physiol.*, **280:** G319–G323.

SURDEANU, M., PENCU, E., TONCIU, M., MIHAI, I. AND CIUDIN, L., 2000. Differentiation of *Shigella* strains by plasmid profile analysis, serotyping and phage typing. *Rom. Arch. Microbiol. Immunol.,* **59:**103-117.

TALUKDER, K. A., ISLAM, M. A., KHAJANCHI, B. K., DUTTA, D. K., ISLAM, Z., SAFA, A., ALAM, K., HOSSAIN, A., NAIR, G. B. AND SACK, D. A., 2003. Temporal Shifts in the Dominance of Serotypes of *Shigella dysenteriae* from 1999 to 2002 in Dhaka, Bangladesh. *J. clin. Microbiol.,* **41**: 5053-5058.

WEISLANDER, L., 1979. A simple method to recover intact high molecular weight RNA and DNA after electrophoretic separation in low gelling temperature agarose gels. *Anal Biochem.,* **98**: 305-309.

YANG, F., YANG, J., ZHANG, X., CHEN, L., JIANG, Y., YAN, Y., TANG, X., WANG, J., XIONG, Z., DONG, J., XUE, Y., ZHU, Y., XU, X., SUN, L., CHEN, S., NIE, H., PENG, J., XU, J., WANG, Y., YUAN, Z., WEN, Y., YAO, Z., SHEN, Y., BOQIN QIANG, B., HOU, Y., YU, J. AND JIN, Q., 2005. Genome dynamics and diversity of *Shigella* species, the etiologic agents of bacillary dysentery. *Nucl. Acids Res.,* **33**: 6445–6458.

(Received 12 July 2008, revised 26 May 2009)