Pakistan J. Zool., vol. 46(6), pp. 1605-1616, 2014.

Plasmid Profile Analysis and Antimicrobial Resistance Pattern of *Shigella flexneri* Strains Isolated From Azad Kashmir, Pakistan

Basharat Ahmed,[1] K. Al- Ghanim,[2] Shahid Mahboob,[2,3] Farah R. Shakoori[4] and A. R. Shakoori[1,5]
[1]*School of Biological Sciences, University of the Punjab, Quaid-i-Azam Campus, Lahore 54590, Pakistan*
[2]*Department of Zoology, College of Science, King Saud University, P.O. Box 2455, Riyadh 11451, Saudi Arabia.*
[3]*Department of Zoology, GC University, Faisalabad, Pakistan*
[4]*Department of Zoology, University of the Punjab, Quaid-i-Azam Campus, Lahore 54590, Pakistan*
[5] *Visiting Professor Department of Zoology, College of Science, King Saud University, P. O. Box 2455, Riyadh 11451, Saudi Arabia*

Abstract.- This study was carried out to determine the antimicrobial susceptibility patterns of 584 *Shigella flexneri* isolated from diarrheal patients admitted in hospitals in Azad Kashmir Pakistan and to evaluate their changing trends against 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. flexneri* isolates were sensitive to cefixime, ciprofloxacin and enoxacin. Multiple drug resistance (MDR) was observed against 3-10 drugs and was resistant against three or more antibiotics was at the 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 32.8% MDR strains of *S. flexneri* which were found resistant against three or more antibiotics. The number of plasmids varied from one to seven. Analysis of plasmid DNA of *S. flexneri* 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. Some of the antibiotic resistance determinants were cured by acridine orange, indicating that widespread antibiotic resistance is mediated through plasmid. Transformation experiments showed that the factors for resistance against ampicillin, chloramphenicol and sulfamethoxazole-trimethoprim resided in >23.1 Kb and 23.1 Kb plasmids.

Key words: *S. flexneri*, antibiotic resistance, R-plasmid, plasmid curing, multiple drug resistance, diarrhoea, Shigellosis,

INTRODUCTION

Shigella is a rod shaped non-motile, nonspore-forming, facultative anaerobic Gram-negative bacterium. It is lactose-fermenting bacterium causing dysentery (Yang *et al.*, 2005). In developing countries shigellosis is widespread and causes extensive outbreaks. In industrialized countries, this disease has become rare, and it currently occurs as sporadic cases in migrant workers or those who travel to developing countries and is limited to epidemic episodes among children in daycare centers, individuals in custodial institutions and homosexual men (Niyogi, 2005). Consequently, although shigellosis is a major public health concern, there is a great disparity between developing countries (over 163.2 million cases each year) and developed ones (1.5 million cases). 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). Dramatic outbreaks may also occur, particularly in the context of humanitarian disasters (wars, refugee camps). Shigellosis is not the most frequent cause of diarrhoeal disease, but its dysenteric form is the most severe; each year, it kills between 600,000 and 1 million people, mostly children in the developing countries. Most that are infected with *Shigella* develop diarrhoea, fever, and stomach cramps starting a day or two after they are exposed to the

* Corresponding author: Prof. A. R. Shakoori, arshakoori.sbs@pu.edu.pk, arshaksbs@yahoo.com
0030-9923/2014/0006-1605 $ 8.00/0

bacterium. Shigellosis usually resolves in 5 to 7 days. In some persons, especially young children and the elderly, the diarrhoea can be so severe that the patient needs to be hospitalized. A severe infection with high fever may also be associated with seizures in children less than 2 years old.

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* bacteria that might be present in the patient's stools, and shortens the illness. Unfortunately, some *Shigella* bacteria have become resistant to antibiotics and using antibiotics to treat shigellosis can actually make the germs more resistant in the future. Persons with mild infections will usually recover quickly without antibiotic treatment. Therefore, when many persons in a community are affected by shigellosis, antibiotics are sometimes used selectively to treat only the more severe cases. Indiscriminate use of the drugs and horizontal gene transfer has led to *Shigella* species becoming resistant to commonly used antibiotics (Noriega *et al.*, 1999). Shigellosis is one of the major diarrhoeal diseases in Bangladesh and several other countries and is responsible for a significant number of deaths, especially among children (Kotloff *et al.*, 1999). In areas, where diarrhoeal disease is endemic, *S. flexneri* is usually the most prevalent species. Until recently, at least 47 serotypes of *Shigella* have been recognized; of which 15 belong to *S. flexneri*. More elaborately, *S. flexneri* has eight serotypes, of which serotypes 1 to 5 are further classified into 12 subserotypes (Talukder *et al.,* 2001). This classification scheme for *S. flexneri* is not comprehensive because atypical strains or newer subserotypes are being isolated from different parts of the world, including Bangladesh (Talukder *et al.*, 2002). Among serotype 1 isolates, the prevalence of provisional serotype *S. flexneri* 1c increased from 0 to 56% from 1978 to 2001 in Bangladesh (Talukder *et al.*, 2003).

The indiscriminate use of antibiotics is considered the main factor in emergence and dissemination of antibiotic resistance. 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. The present study was aimed at determining the status of antibiotic resistance in *S. flexneri* in Azad Kashmir, and to investigate the virulence factors in the local isolates and their possible role in infection.

MATERIALS AND METHODS

Bacterial strains

Shigella flexneri strains were isolated from stools of patients suffering from diarrhoea admitted in 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. For the isolation of *S. flexneri* 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 *Salmonella-Shigella* agar (SSA) and MacConkey agar plates and incubated at 37°C for 24 h. Non-lactose fermenting colonies (*i.e.* colorless) on MacConkey agar plates were inoculated on xylose lysine deoxycxholate 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 to locate Gram negative short rods. All plates were incubated aerobically at 37ºC for 24 h. From amongst the suspected *S. flexneri* from both SSA and MacConkey agar, the non-lactose-fermenting colonies were biochemically identified on urea, triple sugar iron, sulphide-sulphide-indol and motility medium, 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 (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 OXOID, 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). All solutions were sterilized by Millipore (0.45mμ) filters and refrigerated.

Antimicrobial sensitivity testing

Antibiotic susceptibility tests of the collected isolates of *S. flexneri* were performed by antibiotic disc diffusion method (Bauer *et al.,* 1966) using filter paper discs. The minimum inhibitory concentrations (MICs) using a specific range of concentrations of fifteen commonly used antibiotics (25, 50, 100, and 300μg/ml) were determined by agar dilution method. In this study the MIC was defined, as the lowest concentration of antibiotic at which no growth was visible. 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 (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 electrophoresis (Meyers *et al.,* 1976). The plasmid DNA was purified by removal of RNA with the help of RNase. To estimate the size of plasmid DNA, DNA Marker (λ DNA cut with *Hin*d-III) was used. After gel electrophoresis various plasmid DNA bands were cut out of the gel individually, DNA extracted and purified by the usual molecular biological techniques (Weislander, 1979).

Plasmid curing

The method of Hirota (1960) was followed for this purpose. Inocula ($2x10^{-2}$ - $5x10^{-2}$ bacteria) was added to varying concentrations of acridine orange broth and incubated at 37ºC overnight. Culture containing the highest concentration of acridine orange in which growth was clearly visible was diluted and spread on nutrient agar plates containing appropriate antibiotic.

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 MDR *S. flexneri* was added to competent cells of *E. coli* HB101, incubated on ice for 30 min and then at 42ºC for two min. One ml of pre-warmed LB broth was then added to this mixture and re-incubated at 37ºC at 60 rpm for 80 min. The whole mixture was then spread on two different Luria-Bertani agar plates containing ampicillin (100 μg/ml), chloramphenicol (100 μg/ml) sulfamethoxazole-trimethoprim (100 μg/ml) and incubated at 37ºC overnight (Sambrook *et al.,* 1989).

RESULTS

Prevalence vs age groups

In this study, 584 strains of *S. flexneri* were isolated during the study period, 149 (66.2%) were isolated in 2004, 94 (55.9%) in 2005, 125 (65.8%) in 2006, 81 (66.4%) in 2007 and 135 (65.5%) in 2008. *S. flexneri* decreased from 66.2% in 2004 to 65.5% in 2008.

Over all, the highest proportion of stool specimens infected with *S. flexneri* was observed in the age group of 6-10 years and 21-30 years (66.7%) followed by 0-5 years (66.5%), 11-20 years (63.2%), >60 years (46.1%), 51-60 years (45.4%) and 31-40 years (44.1%). The lowest infestation was observed in the age group 41-50 years (42.8%) as against 67% in children of 0-5 years of age.

Antimicrobial sensitivity testing

Five hundred and eighty four clinical isolates of *S. flexneri* were screened for commonly used antibiotics resistance. The resistance pattern of the isolates is shown in Table I. Overall 65.1% *S. flexneri* isolates were resistant to penicillin (P) followed by 56.3% to carbenicillin (Ca), 55.6% to ampicillin (A), 51.9% to tetracycline (T), 50.5% to erythromycin (Er), 47.9% to ceftizoxime (CXM), 43.3% to kanamycin (K), 42.9% to co-trimoxazole (Co), 41.8% to piperacillin (Pa), 39.0% to amoxicillin (Am), 37.8% to amikacin (Ak), 31.2% to streptomycin (S), 29.3% to nalidixic acid (Na), 27.4% to gentamicin (G), 26.1% to chloramphenicol

Table I.- Occurrence of antibiotics resistance of 584 *S. flexneri* isolates at four different concentrations.

Antibiotics	No. of resistant isolates at			
	25 µg/ml	50 µg/ml	100 µg/ml	300 µg/ml
Amikacin (Ak)	221(37.8%)	186 (31.8%)	82 (14.0%)	16 (2.7%)
Ampicillin (A)	325(55.6%)	270 (46.2%)	164 (28.1%)	71 (12.1%)
Amoxicillin (Am)	228(39.0%)	197 (33.7%)	88 (15.0%)	13 (2.2%)
Carbenicillin (Ca)	329(56.3%)	304 (52.0%)	187 (32.0%)	75 (12.8%)
Cefixime (Cef)	00(0.0%)	00 (0.0%)	00 (0.0%)	00 (0.0%)
Ceftizoxime (CXM)	280(47.9%)	252 (43.1%)	141 (24.1%)	52 (8.9%)
Ceftriaxone (Cz)	114(19.5%)	87 (14.9%)	36 (6.2%)	3 (0.5%)
Cephalothin (Cl)	130(22.3%)	118 (20.2%)	40 (6.8%)	7 (1.2%)
Chloramphenicol (C)	153(26.1%)	132 (22.6%)	51 (8.7%)	12 (2.0%)
Ciprofloxacin (Cip)	00(0.0%)	00(0.0%)	00 (0.0%)	00 (0.0%)
Co-trimoxazole (Co)	251(42.9%)	229 (39.2%)	109 (18.6%)	20 (3.4%)
Enaxacin (E)	00(0.0%)	00 (0.0%)	00 (0.0%)	00 (0.0%)
Erythromycin (Er)	294(50.3)	280 (47.9%)	155(26.5%)	56 (9.6%)
Gentamicin (G)	160(27.4%)	145 (24.8%)	43 (7.4%)	4 (0.7%)
Kanamycin (K)	253(43.3%)	232 (39.7%)	170 (29.1%)	46 (7.9%)
Nalidixic acid (Na)	171(29.3%)	151 (25.8%)	54 (9.2%)	16 (2.7%)
Penicillin (P)	380(65.1%)	353(60.4%)	266 (45.5%)	112 (19.2%)
Sulfamethoxazole-	244(41.8%)	210 (35.9%)	95 (16.3%)	19 (3.2%)
Trimethoprim (SxT)	182(31.2%)	159 (27.2%)	64 (10.9%)	17 (2.9%)
Streptomycin (S)	303(51.9%)	293(50.2%)	168 (28.8%)	60 (10.3%)
Tetracycline (T)				

(C), 22.3% to cephalothin (Cl), and 19.5% to ceftriaxone (Cz). All *S. flexneri* isolates were sensitive to cefixime (Cfm), ciprofloxacin (CIP) and enoxacin (E).

Table I shows resistance of 584 strains of *S. flexneri* against twenty antibiotics at four concentrations *viz.,* 25, 50, 100 and 300µg/ml. Generally, the isolates showed the highest resistance against penicillin and the lowest resistance was against ceftriaxone at all the four levels of antibiotics screened. At 300µg/ml the isolates showed considerable decrease in the resistance frequency of almost all the antibiotics tested.

MDR was observed in this study ranging from three to ten drugs. Out of 584 isolates, screened for antibiotic resistance, 36% were resistant at 25µg/ml, 32% were resistant at 50µg/ml, 10% were resistant at 100µg/ml and 5% were resistant at 300µg/ml to three or more antibiotics. The resistant isolates showed different patterns of antibiotics resistance. The most common pattern was PCaA at all the four levels shown in Table II.

Plasmid profile

A total of 225 isolates of *S. flexneri* were

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

Antibiotics resistance patterns	Percent of resistant isolates at (µg/ml)			
	25	50	100	300
P, Ca, A	36	32	10	5
P, A, T	33	30	9	4
P, Ca, T	30	26	7	4
P, Ca, A, T	24	22	7	3
P, A, T, Er	21	18	5	3
P, Ca, A, Er	17	31	5	2
P, Ca, A, CXM	15	11	5	2
P, Ca, A, T, Er	12	10	4	1
P, C, A, T, CXM	9	6	4	1
P, Ca, T, CXM, K	7	5	3	1
P, Ca, A, T, K, Co	5	5	2	1
P, A, T, Er, K, Co	4	3	2	-
P, Ca, A, T, Er, Co, SxT, Am	3	2	1	-
P, A, C, Er, K, Co, Am, Ak, S, Na	2	1	-	-
P, Ca, A, T, Co, Am, Ak, S, Na, G	1	1	-	-

Key: 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.

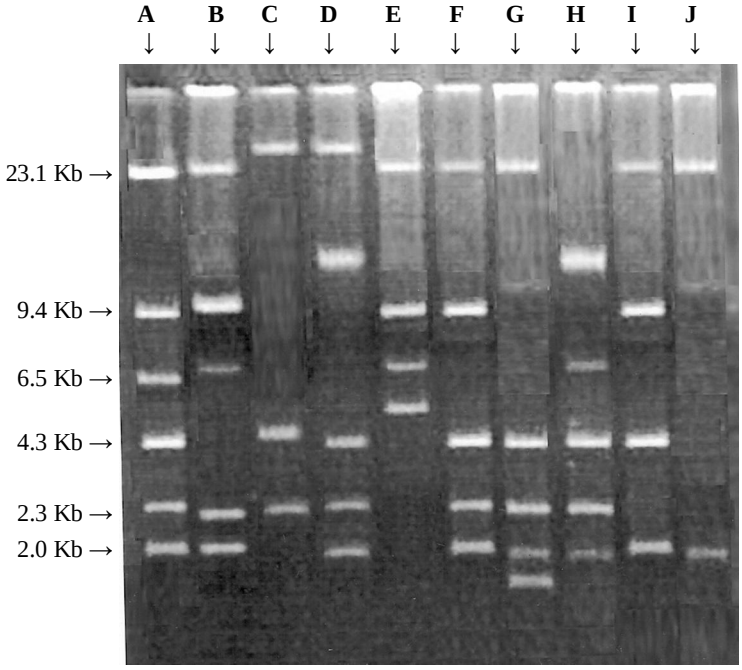

Fig. 1. Plasmid profile of representative *S. flexneri* strains isolated from fecal samples of patients with gastroenteritis in Azad Kashmir. Lane A, marker λ DNA cut with *Hind* III; Lane B, BSf-415; Lane C, BSf-418; Lane D, BSf-475; Lane E, BSf-480; Lane F, BSf-596; Lane G, BSf-4072; Lane H, BSf-4127; Lane I, BSf-4162; Lane J, BSf-4291). [in BSF, B is author's name; Sf is *S. flexneri* and digits are sample numbers].

processed for isolation of plasmids and only 74 (32.8%) carried plasmids. These isolates were resistant to three or more antibiotics used in this study. The number of plasmids varied from one to seven.

In *S. flexneri*, the analysis of plasmid DNA revealed that all the isolates contained a heterogeneous population of plasmids ranging between >23.1 kb to <2.0 kb (Fig.1). The most dominant plasmids were 2.0 Kb (82.4%), 2.3 Kb (67.6%), 23.1 Kb (56.7%), 4.3 Kb (44.6%), 6.5 Kb (40.5%), >23.1 Kb (36.5%) and 4.3 Kb (32.4%). Other plasmids were observed in lesser frequency. The frequency of 9.4 Kb plasmid was 25.7%, for >9.4 Kb it was 22.9% and for <2.0 Kb it was 8.1%.

Based on molecular weight, the pattern of different plasmids was also very diverse. Depending upon the number of plasmids, the 74 individual strains were grouped into nine different plasmid patterns, designated P1-P9. Sixteen isolates (21.6%) had pattern P1 (5 plasmids), fifteen isolates (20.3%) had pattern P2 (3 plasmids), twelve isolates (16.2%) had pattern P3 (5 plasmids), nine isolates (12.2%) had pattern P4 (4 plasmids), seven isolates (9.4%) had P5 (5 plasmids), six isolates (8.1%) had P6 (5 plasmids), five isolates (6.7%) had P7 (5 plasmids), three isolates (4.0%) had P8 (4 plasmids) and the remaining one strain (1.3%) had pattern P9 (2 plasmids) (Table III).

Antimicrobial resistance determinants are plasmid borne

Plasmid curing

Representative MDR isolates of *S. flexneri* (BSf-475, BSf-4072 and BSf-4291) were selected for plasmid curing. Acridine orange was used as curing agent during this study for elimination of plasmids. Out of 100 colonies each from four treated cultures some had lost the resistance to one or the other antibiotic. Effect of plasmid curing on the drug resistance determinants of *S. flexneri* isolates is shown in Table IV.

Transformation

Of the 74 *S. flexneri* isolates, the plasmids of 35 strains were used for transformation of *E. coli*

Table III.- Plasmid profile analysis of 74 isolates of *S. flexneri.*

No of isolates	Plasmid (Kb)										Plasmid pattern
	>23.0	23.1	>9.4	9.4	6.5	>4.3	4.3	2.3	2.0	<2.0	
16	-	+	-	+	+	-	-	+	+	-	P1
15	+	-	-	-	-	-	+	+	-	-	P2
12	+	-	+	-	-	-	+	+	+	-	P3
9	-	+	-	+	+	+	-	-	-	-	P4
7	-	+	-	+	-	-	+	+	+	-	P5
6	-	+	-	-	-	-	+	+	+	+	P6
5	-	-	+	-	+	-	+	+	+	-	P7
3	-	+	-	+	-	-	+	-	+	-	P8
1	-	+	-	-	-	-	-	-	+	-	P9

Table IV.- Effect of acridine orange mediated plasmid curing on the antibiotic resistance pattern of *S. flexneri* isolates.

Isolate no.	Resistance pattern	
	Pre-curing	Post-curing
BSf-475	PCaA	-
BSf-4072	PCATCXM	-
BSf-4290	PCAKCoSxTAmAk	Am
BSf-4291	PACErKCoAmAkSNa	AmS

HB101 separately ascertaining ampicillin (MIC-100 µg/ml), chloramphenicol (MIC-100 µg/ml) and sulfamethoxazole-trimethoprim (MIC-100 µg/ml) sensitivity. Plasmids of 23 (65.7%), 19 (54.3%) and 17 (48.6%) isolates were used for transformation of *E. coli* HB101 and tested for ampicillin, chloramphenicol, and sulfamethoxazole-trimethoprim resistance, respectively. Of the 35 transformations, 30 (85.7%) were successfully accomplished as *E. coli* HB101 acquired antibiotic resistance to ampicillin, chloramphenicol and sulfamethoxazole-trimethoprim. Plasmids of three isolates (BSf-415, BSf-596, and BSf-4127) were successfully transferred to *E. coli* HB101 shown by the acquisition of resistance to ampicillin, and plasmids of another three strains (no. BSf-418, BSf-480 and BSf-4162) with chloramphenicol resistance were also successfully introduced into *E. coli* HB101. Plasmids of 23 isolates resistant to ampicillin, 19 isolates resistant to chloramphenicol, and 17 isolates resistant to sulfamethoxazole-trimethoprim were also successfully introduced into *E. coli* HB101.

Table V.- Transformation of plasmids of *S. flexneri* into *E. coli* Hb101.

Sample no.	No. of plasmids	Molecular weight of plasmids which were individually transferred to *E. coli* HB101.	Transformed plasmids that conferred antibiotic resistance.
475	5	>23.1Kb, >9.4Kb, 4.3Kb, 2.3Kb, 2.0Kb.	>23.1Kb
4072	**5**	23.1Kb, 4.3Kb, 2.3Kb, 2.0Kb, <2.0Kb.	23.1Kb
2091	**2**	23.1Kb, 2.0Kb	23.1Kb

In some multiple plasmid isolates (no. BSf-475, BSf-4072 and BSf-4291), 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 V).

DISCUSSION

Prevalence vs age group

Shigellosis is primarily a childhood disease in both developed and developing countries including Pakistan, whereas epidemic shigellosis affects all age groups (Keusch and Bennish, 1991; Ahmed and Shakoori, 1996). The information about the etiology and drug sensitivity pattern of bacterial strains is however, lacking due to the lack of diagnostic facilities. In this study, the prevalence *S. flexneri* decreased over the years from 66.2% in 2004 to 65.5% in 2008. The highest proportion of stool specimens infected with *S. flexneri* was observed in the age group of 6-10 years. Lowest infestation was observed in the age group 41-50 years (42.8%). Almost similar results were reported by earlier workers (Ahmed and Shakoori, 2003). Khalil *et al.* (1998) reported slightly higher prevalence in the age groups of 11-20 and 21-30 years. The highest infestation of *Shigella* was observed 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 Eastern Nepal. The high incidence of shigellosis in children is attributed to their poor resistance, lack of previous exposure, poor hygiene, and higher exposure to contaminated environment due to play-related activities. In the present study, the higher incidence of shigellosis in the age groups of 6-10 years is attributable to poor hygiene and fecal matter contaminated environment. In the areas where diarrhoeal disease is endemic, *S. flexneri* is usually the most prevalent species (Henry, 1991). Until recently, at least 47 serotypes of *Shigella* were recognized, of which 15 belonged to *S. flexneri* (Clemens *et al.*, 1999).

Antibiotic resistance

The use of antimicrobial agents in the treatment of diarrhoea has greatly improved the quality of life among residents in and travelers to developing countries (Tjaniadiu *et al.*, 2003). Development of resistance has however, complicated the therapeutic activities. In the current study, 65% *S. flexneri* isolates were resistant to penicillin followed by 56.3% to carbenicillin,, 55.6% to ampicillin, 51.9% to tetracycline, 50.5% to erythromycin, 47.9% to ceftizoxime, 43.3% to kanamycin, 42.9% to co-trimoxazole, 41.8% to piperacillin, 39.0% to amoxicillin, 37.8% to amikacin, 31.2% to streptomycin, 29.3% to nalidixic acid, 27.4% to gentamicin, 26.1% to chloramphenicol, 22.3% to cephalothin, and 19.5% to ceftriaxone. All *S. flexneri* isolates were sensitive to cefixime, ciprofloxacin and enoxacin. These results are comparable with the results of a previous study in Iran (Nowroozi and Hakemivala, 2006), where it was reported that 56.7% of isolated *S. flexneri* strains had multi drug resistance to ampicillin, tetracycline, and trimethoprime-sulfamethoxazole, but 100% of isolates were sensitive to ciprofloxacin. Similar results have been reported by Turner *et al.* (2003) where 100% of *S. flexneri* isolates were resistant to carbenicillin, streptomycin, chloramphenicol, tetracycline, ampicillin, ticarcillin. However, 39.3% isolates were resistant to trimethoprime-sulfamethoxazole and 6% were resistant to nalidixic acid. All the strains were susceptible to cephalotin, colistin, kanamycin, amikacin, ciprofloxacin, cefoxitin and cefotaxime. Results of this study showed more resistance to tetracycline, ampicillin, and trimethoprime-sulfamethoxazole, which might be due to geographic differences or indiscriminate use of these drugs. In another study, Kaisar *et al.* (2003) showed that 42% of isolates were resistant to three commonly used antibiotics, ampicillin, tetracycline and trimethoprime-sulfamethoxazole, which was close to our result.

In their study, Casalino *et al.* (1994) reported that all but three of the strains were resistant at least to ampicillin, chloramphenicol, spectinomycin, and tetracycline. Of these resistant strains, 41 were resistant to sulfonamide and streptomycin and 14 were resistant to trimethoprim or trimethoprim and kanamycin. Similarly, in Bangladesh Talukder *et al.* (2003) reported that, all *S. flexneri* serotype 1b were resistant to tetracycline, 94% were resistant to ampicillin, 72% were resistant to trimethoprim-sulfamethoxazole, and 18% were resistant to nalidixic acid. None of the strains were resistant to mecillinam or ciprofloxacin. In a study in southeast Brazil, Penatti *et al.* (2007) reported that the *S. flexneri* strains were resistant to ampicillin in 83.3%

of cases, chloramphenicol in 70.0%, streptomycin in 86.7%, sulfamethoxazole in 80.0%, and tetracycline in 80.0%, while a smaller number of strains were resistant to cephalothin (3.3%) and sulfazotrim (10.0%). In southren Ethiopia, out of the ninety-nine *S. flexneri* group B strains isolated, 95 were found to be susceptible to gentamicin and 90 to nalidixic acid. In addition, 55.5% of *S. flexneri* strains were reported to be resistant to trimethoprim-sulfamethoxazole (Cobra and Sack, 1996).

In the current study, the isolates showed the highest frequency of resistance against penicillin at all concentrations of antibiotics used. The lowest resistance was against ceftriaxone followed by cephalothin and chloramphenicol. The resistance of *S. flexneri* to doses as high as 300µg/ml is alarming, because, if *S. flexneri* become resistant to such high levels of antibiotics, the treatment of disease with antibiotics would become quite difficult. In Pakistan Ahmed and Shakoori (1996) reported highest frequency of resistance against septran at 50 and 100µg/ml whereas chloramphenicol resistance was 88.8%. In another study in Pakistan (Ahmed and Shakoori, 2001), documented 50% resistance of *Shigella* strains and in Northern Areas of Pakistan Ahmed *et al.* (2003) reported 14.3% resistance of *Shigella* strains against chloramphenicol. MDR was observed in this study ranging from three to ten drugs. The most common pattern was PCaA at all the four concentrations. Analogous results were reported by other investigators in many countries including Pakistan (Ahmed and Shakoori, 2001; Ahmed *et al.*, 2003).

Plasmid borne resistance factors

There are large numbers of antimicrobial agents such as penicillin, cephalosporin, tetracycline, spectinomycin, chloramphenicol, fusidic acid, sulfonamides, heavy metal and others for which plasmid-mediated antibiotic resistance has been reported. The cause of the increase in R factor-carrying bacteria is due to the selective pressure caused by antibiotics and other chemotherapeutic agents. These drugs are currently being used not only in humans, but also in animals, cultured fish, fruits, vegetables, rice plants, and honey bees. It has been shown that the use of antibiotics in animal and fish culturing greatly increase the pool of R factor-carrying bacteria in the environment. It seems likely that the use of antibiotics for other non-medical purposes also helps the increase of the reservoir of R factors (Watanabe, 1972). A high incidence of plasmid-mediated resistance has been observed in enterobacteria including *Shigella* in many countries. The most common pattern was resistance to sulfonamide and streptomycin, which were carried by a single plasmid. The strains of *Shigella* resistant to various commonly used antibiotics have been reported from various parts of the world including Pakistan (Ahmed and Shakoori, 2001; Ahmed *et al.*, 2003). Multiple drug resistance in *Shigella* has complicated the situation in recent years (Ghosh and Sehgal, 1998). The genes for resistance to ampicillin, chloramphenicol, spectinomycin, and tetracycline formed a linkage group located on the chromosome of the strains of all serotypes (Casalino *et al.*, 1994).

Plasmid profile

Plasmid profile analysis is a useful tool in epidemiological studies dealing with enteric infections. In the present work the multiple drug resistant (MDR) strains of *S. flexneri* were processed for isolation of plasmids. This study revealed that (32.8%) strains of *S. flexneri* carried plasmids. These were found resistant to three or more antibiotics. The number of plasmids varied from one to seven. *Shigella* spp. is known to carry as many as 10 plasmids (Ling *et al.*, 1993), these results indicate a high probability of identity between the R-plasmids found in *S. flexneri* which usually encoded resistance to ampicillin, tetracycline, chloramphenicol, sulfamethoxazole-trimethoprim and co-trimoxazole. Genes for resistance to sulfonamide and streptomycin were located on a 63-kb plasmid in strains of *S. flexneri* serotypes lb, 2a, and 4 (Casalino *et al.*, 1994). The large plasmid was found to contain a gene conferring virulence (Lin and Chang, 1992), the large plasmid was very unstable and easily lost, only a small number of strains of *Shigella* species were found to have the large virulence plasmid (Vargas *et al.*, 1999).

Shigella with plasmid–mediated multiple

drug resistance, including Pakistan (Ahmed and Shakoori, 2001; Ahmed *et al.,* 2003) have been well documented in India (Dutta *et al.,* 2002) and in Bangladesh (Talukder *et al.,* 2003; Iqbal *et al.,* 2014). In the present study, all the MDR isolates of *S. flexneri* 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 and were found among (MDR) *S. flexneri* strains. Similar results are reported in a previous study in Iran (Nowroozi and Hakemvala, 2006), where they reported that eleven distinct plasmid patterns were identical and plasmid size ranged from 0.564 kb to 21.226 kb. Plasmids of the same size were present in multiple strains, for example, most of strains (78.8%) harbored the 21.226 kb plasmid. Similarly the comparable results have been presented in a previous study, Hoe *et al.* (2005) reported that the heterogeneous plasmid patterns were observed in all *Shigella* spp. There was a correlation between plasmid patterns and serotypes of *S. flexneri*, *S. dysenteriae* and *S. sonnei*. Five common small plasmids (>20.0 kb) were observed in *S. flexneri* 1b and 2a, whereas six common small plasmids were found in serotype 3a. Some of these plasmids appeared to maintain their existence stably in each individual serotype. Plasmids of size 11.40 and 4.20 kb were present only in *S. flexneri* 2a isolates, whereas the 4.40 kb plasmid was unique for serotype 3a. Large (>150 kb) or mid-range plasmid (20.0–150 kb) was not observed from any *S. flexneri* 1b isolates. Eighty-nine percent of *S. flexneri* of various serotypes harbored the plasmid of 3.20 kb. Similarly, as reported in a previous study, that the plasmid profiles of *S. dysenteriae* serotype 1, *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. Each of the serotypes presented with unique plasmid profile, hence this can be used as an epidemiological marker tool for strain differentiation.

Location of drug resistance determinant

The location (chromosomal or extra chromosomal) of drug resistance determinants was also confirmed by plasmid curing strategies. In this connection, acridine orange mediated curing was performed. The loss of resistance to single or multiple antibiotics after acridine orange treatment of isolates, point to the fact that observed antibiotic resistance was plasmid borne. In the present study, the representatives multiple drugs resistant isolate of *S. flexneri* were selected for plasmid curing. Acridine orange was used as curing agent during this study for the elimination of plasmid. Out of 100 colonies each from treated cultures some had lost the resistance to one or the other antibiotic. Effects of plasmid curing on the drug resistance determinants of *S. flexneri* isolates are depicted in (Table V). Resultantly, some of the resistance markers were stably lost (excluding amoxicillin and streptomycin in terms of the MDR *S. flexneri* strains; thereby showing the chromosomal location of these two markers). However, a total loss to penicillin was found in the cultures. Similar studies were performed by earlier workers (Joan, 1997; Rasool *et al.,* 2003) where some of the representative isolates lost the antibiotic resistance after acridine orange mediated curing. The resistance markers were stably lost (excluding amoxicillin and streptomycin in terms of the MDR *Klebsiella* strains; there by showing the chromosomal location of these two markers.

In many cases, resistance genes are found to reside on easily transferable R-plasmids and various studies report that between 45 and 73% of these plasmids are transferable (Ling *et al.,* 1993; Hoe *et al.,* 2005). In current study, the plasmids of MDR *S. flexneri* strains were processed for transformation into *E. coli* HB101 separately for ampicillin (MIC-100 µg/ml), chloramphenicol (MIC-100 µg/ml) and sulfamethoxazole-trimethoprim (MIC-100 µg/ml. The transformations of (85.7%) were successfully accomplished as *E. coli* HB101 acquired antibiotic resistance to ampicillin, chloramphenicol and sulfamethoxazole-trimethoprim. 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. These results are comparable with results of a previous study in Somalia where Casalino *et al.* (1994) reported that the multiple

drug resistance had become widely disseminated in the *S. flexneri* population, with a high rate (41%) of resistance to five or more drugs. The components of the resistance patterns mediated by plasmids were the resistance to SU and SM and the resistance to TP or TP and KM. Conjugative trimethoprim or trimethoprim and kanamycin resistance plasmids with lengths of 80 to 110 kb were present.

In conclusion, the present study demonstrates that *S. flexneri* is the predominant species. There is a significant increase in resistance to several commonly-used antimicrobial agents. The results suggest reconsideration of the empiric use of these antimicrobial agents for the treatment of shigellosis. Our findings stress the need for distributing reliable information about antimicrobial resistance patterns and for ongoing drug resistance surveillance. Search for alternate new drugs should be continued because, although newer antimicrobial drugs can offer hope for treatment of shigellosis, emergence of resistance to the new drugs is also not far in the future. Thus, intensive water and sanitation programs and vaccine development would seem to be critical. The preventive measures need to be directed not only at the prudent use of antimicrobial agents but also at risk factors such as preparation of food, storage and treatment of water, and interruption of person-to-person spread. It is emphasized that hands should be washed before eating, before feeding children, after defecation and after disposal of children's excreta. These measures are further reinforced in epidemic situations, when because of the very low infective dose of the organism and its potential for rapid spread, stringent control measures need to be instituted through simple but effective health education messages to the common masses.

ACKNOWLEDGEMENT

The authors would like to express their sincere appreciation to the Deanship of Scientific Research at the King Saud University for funding of this research through the Research Group Project No. RGP- 341.

Conflict of interest statement

The authors have no conflict of interest to declare.

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, 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., SHERRIS, J.C. AND TURCK, M., 1966. Antibiotic susceptibility testing by a standard single disk method. *Am. J. clin. Pathol.*, **45**: 493-496.

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.

CASALINO, M., NICLETTI, M., SALVIA, A., COLONNA, B., PAZZANI, C., CALCONI, A., MOHAMUD, K.A. AND MAIMONE, F., 1994. Characterization of endemic *Shigella flexneri* strains in Somalia: antimicrobial resistance, plasmid profiles, and serotype correlation. *J. clin. Microbiol*., **32**: 1179-1183.

CLEMENS, J.D., KOTLOFF, K. AND KAY, B.A., 1999. *Generic protocol to estimate the burden of* Shigella diarrhoeaa *and dysenteric mortality.* World Health Organization, Geneva, Switzerland, WHO/ VandB/99. 26.

COBRA, C. AND SACK, D.A., 1996. The control of epidemic dysentery in Africa: Overview, recommendations and checklists. Health and human resources analysis for Africa. *Technical paper No.37; 1996.*

DUTTA, S., RAJENDRAN, K., ROY, S., CHATTERJEE, A., DUTTA, P., NAIR, G.B., BHATTACHARYA, S.K. AND YOSHIDA, S.I., 2002. Shifting serotypes, plasmid profile analysis and antimicrobial resistance pattern of *shigella* strains isolated from Kolkata, India, during 1995-2000. *Epidemiol. Infect.,* **129**: 235-243.

GHOSH, A.R. AND SEHGAL, S.C., 1998. *Shigella* infections among children in Andaman and archipelage of tropical islands in Bay of Bengal. *Epidemiol. Infect.,* **121**: 43-48.

HENRY, F.J., 1991. The epidemiologic importance of dysentery in communities. *Rev. Infect. Dis*., **13**(Suppl. 4):S238-S244.

HIROTA, Y., 1960. The effect of acridine dyes on mating type factors in *Escherichia coli. Proc. natl. Sci. USA*, **46**: 57-64.

HOE, C.H., R.M., 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. Biotech.,* **21**: 271-78.

JOAN, S., 1997. Worry grows as antibiotic resistant bacteria continue to gain ground. *J. Am. med. Assoc.,* **278**: 2049-2050.

KAISAR, A., ZHAHIRUL ISLAM, T. AND AMINUL ISLAM, M., 2003. Phenotypic and genotypic characterization of provisional serotype *S. flexneri 1c* and clonal relationship with *1b* strains isolated in Bangladesh. *J. clin. Microbiol.,* **41**: 110-117.

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.

IQBAL,M.S., RAHMAN, M., ISLAM, R., BANIK, A., AMIN, M.B., AKTER, F. AND TALUKDER, K.A., 2014. Plasmid mediatedsulfa-methoxazole resistance encoded by the sul2 gene in the multi drug-resistant *Shigella flexneri 2a* isolated from patients with acute diarrhea in Dhaka, Bangladesh. *PloS One*, 9: e85338. Doi:10.1371/Journal.pone.0085238.

KOTLOFF, K.L., WINICKOFF, J.P., IVANOFF, B., CLEMENS, J.D., SWERDLOW, D.L., SANSONETTI, P.J., ADAK, G.K. AND LEVINE, M.M., 1999. Global burden of *Shigella* infections: Implications for vaccine development and implementation of control strategies. *Bull. WHO*, **77**: 651-666.

LIN, S.R. AND CHANG, S.F., 1992. Drug resistance and plasmid profile of Shigellae in Taiwan. *Epidemiol. Infect.,* **108**: 87-97.

LING, J.M., SHAW, P.C., KAM, K.M., CHENG, A.F. AND FRENCH, G.L., 1993. Molecular studies of plasmids of multiply-resistant *Shigella* spp., in Hong Kong. *Epidemiol. Infect*., **110**: 437-446.

MEYERS, J.A., SANCHEZ, D., ELWELL, L.P., AND FALKOW, S., 1976. Simple agarose gel electrophoresis method for the identification and characterization of plasmid deoxyribonucleic acid. *J. Bact.,* 127: 1529-1537.

NATIONAL COMMITTEE FOR CLINICAL LABORATORY STANDARDS. 1993. *Performance standards for antimicrobial disk susceptibility tests*. Document M2-A4, 1993; 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. Immunol.,* **67**: 782-788.

NOWROOZI, J. AND HAKEMI VALA, M., 2006. Plasmid profile, antibiotic resistance, and phenotypic virulent strains of *S. flexneri. Iranian J. Publ. Hlth.,* **35**: 43-48.

PENATTI, M.P.A., HOLLANDA, L.M., NAKAZATO, G., CAMPOS, T.A., LANCELLOTTI, M., ANGELLINI, M., BROCCHI, M., ROCHA, M.M.M. AND Dias Da SILVEIRA,W., 2007. Epidemiological characterization of resistance and PCR typing of *Shigella flexneri* and *Shigella sonnei* strains isolated from bacillary dysentery cases in Southeast Brazil. *Braz. J. Med. biol. Res.,* **40**: 249-258.

RASOOL, S.A., AHMAD, A., KHAN, S AND WAHAB., A., 2003. Plasmid borne antibiotic resistance factors among indigenous *Klebsiella. Pak. J. Bot.,* **35**: 243-48.

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

TALUKDER, K.A., DUTTA, D.K., SAFA, A., ANSARUZZAMAN, M., HASSAN, F., ALAM, K., ISLAM, K.M.N., CARLIN, N.I.A. NAIR, G.B. AND SACK, D.A., 2001. Altering trends in the dominance of *Shigella flexneri* serotypes and emergence of serologically atypical *S. flexneri* strains in Dhaka, Bangladesh. *J. clin. Microbiol.*, **39**:3757-3759.

TALUKDER, K.A., ISLAM, M.A., DUTTA, D.K., HASSAN, F., SAFA, A., NAIR, G.B. AND SACK, D.A., 2002. Phenotypic and genotypic characterization of serologically atypical strains of *Shigella flexneri* type 4 Iiolated in Dhaka, Bangladesh. *J. clin. Microbiol*., **40**: 2490-2497.

TALUKDER, K.A., ISLAM, M.A., KHAJANCHI, B.K., DUTTA, D.K., ISLAM, Z., SAFA, A., ALAM, K., HOSSAIN, M. A., FARUQUE, A.S.G., 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.

TJANIADI, P., LESMANA, M., SUBEKTI, D., MACHPUD, N., KOMALARINI, S., SANTOSO, W., SIMANJUNTAK, C.H., PUNJABI, N., CAMPBELL, J.R., ALEXANDER, W.K., BEECHAM III, H.J., CORWIN, A.L. AND OYOFO, B.A., 2003. Antimicrobial resistance of bacterial pathogens associated with diarrheal patients in Indonesia. *Am. J. trop. Med. Hyg.,* **68**: 666–670.

TURNER, S.A., LUCK, S.N., SAKELLARIS, H., RAJAKUMAR, K. AND ADLER, B., 2003. Molecular epidemiology of the SRL pathogenicity island. *Antimicrob Agents Chemother*., **47**: 727–734.

VARGAS, M., GASCON, J., JIMENEZ DE ANTA, M. T. AND VILA, J., 1999. Prevalence of *Shigella* enterotoxins 1 and 2 among *Shigella* strains isolated from patients with traveler's diarrhea. *J. clin.*

Microbiol., **37:** 3608–3611.

WATANABE, T., 1972. Further outlooks of antibiotics in the shadow of resistance factors. In: *Bacterial plasmids and antibiotic resistance* (eds. V. Kremery, L. Rosival and T. Watanabe). Avicenum, Czechoslovak Medical Press, Prague, pp.9–10.

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 23 May 2014, revised 8 August 2014)