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Antimicrobial Resistance Pattern and Plasmid Analysis of *Escherichia coli* from Patients Suffering from Acute Diarrhoea in Azad Kashmir, Pakistan*

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Abstract.- The antimicrobial susceptibility patterns of 1210 *Escherichia coli* isolated from diarrhoeal patients admitted to hospitals in Azad Kashmir, Pakistan were analyzed from 1994 to 1998 to determine their changing trends in response to fifteen antibiotics. The isolates showed highest resistance against carbenicillin followed by ampicillin, ceftizoxime, co-trimoxazole, streptomycin, amoxicillin, amikacin, tetracycline, erythromycin, nalidixic acid and chloramphenicol. The isolates showed least resistance against ciprofloxacin followed by ceftriaxone and gentamicin. All *E. coli* isolates were sensitive to cefixime. Majority (58%) of *E. coli* strains were recovered from children and 57% were from male patients. Most of the diarrhoeal cases were recovered in summer (36.4%) followed by 35.9% in autumn, 17.7% in spring, and 9.8% in winter. The highest number of stool specimens infected with *E. coli* (40.8%) was recovered in 1998 and the lowest (21.8%) in 1995. It was also observed that the percentage of isolates resistant to any of the antibiotics tested was higher in children than in adults. Resistance of *E. coli* isolates to 3-10 antibiotics was recorded at different concentrations: 49% were resistant at 25µg/ml, 46% at 50µg/ml, 23% at 100µg/ml and 10% were resistant to three or more antibiotics at 300µg/ml. The most common antibiotics resistance pattern was CaACXM. The plasmids were observed in 31.2% strains of *E. coli* resistant to three or more antibiotics. The number of plasmids varied from one to five. Analysis of plasmid DNA of *E. coli* revealed that all the strains contained a heterogeneous population of plasmids ranging between 23.1 kb to 2.0 kb and were grouped into nine different plasmid patterns. The 23.1 Kb plasmid could only confer ampicillin, chloramphenicol and tetracycline resistance to the competent cells of *E. coli* HB101.

Key words: *Escherichia coli*, antibiotic resistance, plasmid borne resistance, Azad Kashmir Pakistan.

INTRODUCTION

Escherichia coli is an important opportunistic pathogen that has shown an increasing antimicrobial resistant to most antibiotics (Miranda *et al.*, 2004) isolated from humans and animals (Poppe *et al*., 2005; Sayah *et al*., 2005). It is a Gram negative bacillus that lives in the intestinal tract of virtually everyone. Intestinal strains of *E. coli* are primary cause of urinary tract infections, septicemia, diarrhoea, neonatal meningitis and nosocomial infections. *E. coli* have been reported to be the leading cause of diarrhoea in addition to pathogens such as *Salmonella, Shigella, Yersinia, Vibrio, Campylobacter* sp., *Entamoeba histolytica*, and *Giardia lamblia* in developing countries. *E. coli* strains causing diarrhoea world wide, mostly in infants include entero-pathogenic *E. coli* (EPEC) entero-toxigenic *E. coli* (ETEC), entero-hemorrhagic *E. coli* (EHEC), entero-invasive *E. coli* (EIEC), entero-aggregasive *E. coli* (EAEC) and entero-adherent *E. coli* (EAEC) or diffusely adhering *E. coli* (DAEC) (Burton and Paul, 2000).

E. coli is the most common cause of Gram negative bacillus infections and have a relatively large potential for developing resistance. Indeed, antimicrobial resistance to β-lactams and other antibiotics has been reported from many countries (Fluit *et al*., 2000). Resistance to fluoroquinolones has been emerging in recent years, even in countries where antimicrobial resistance rates are low, and multidrug resistance has been reported (Sahm *et al*., 2001). Although quinolone resistance results mostly from chromosomal mutations, it may also be mediated by a plasmid-encoded *qnr* gene in members of the family Enterobacteriaceae. A single *E. coli* isolate that carried a ca.180-kb conjugative plasmid encoding a *qnr* determinant has been identified.

However, antimicrobial resistance in enteric

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pathogens complicates the situation in developing countries, where acute diarrhoea is endemic and indiscriminate use of antimicrobial agents is common. In order to ensure appropriate treatment, continual surveillance is required to determine the efficacy of antibiotics used. 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 work is aimed at investigating the virulence factors in the locally isolated *E. coli* and their possible role in infection. The strains of *E. coli* resistant to commonly used antibiotics have been screened for plasmid DNA. The plasmid-less *E. coli* HB101 strains have been transformed with MDR plasmid to determine the location of antibiotic resistance gene.

MATERIALS AND METHODS

Bacterial strains

E. coli strains were isolated from stools of children (0-5 years) and adults, both male and female, suffering from diarrhea, and admitted at different hospitals of Azad Kashmir (Pakistan), over the past 5 years. A questionnaire seeking information on age, sex, address, patient code number and laboratory result was used to collect the data. A loop full of stool specimen was streaked on MacConkey agar plates and incubated at 37°C for 24 h. Pinkish colonies with 2-3 mm in diameter and Gram negative cells were observed. Identification was done by biochemical tests. Identified *E. coli* isolates were then serogrouped using specific grouping antisera (Welcome Diagnostics, Dartford, UK). Only one strain per sample was kept for further studies. Bacterial cultures were maintained in glycerol LB media at -20°C. For routine experiments, the cultures were maintained on LB agar plates at 4°C and subcultured bimonthly.

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 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), chloramphenicol (C), ciprofloxacin (Cip), co-trimoxazole (Co), erythromycin (Er), gentamicin (G), nalidixic acid (Na), streptomycin (S) 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 *E. coli* were performed by antibiotic disc diffusion method (Bauer *et al*., 1966) using filter paper discs. The minimum inhibitory concentrations (MICs) of fifteen commonly used antibiotics at different concentration (25, 50, 100 and 300µg/ml) were determined by agar dilution method. Reference strains *Escherichia coli* ATCC 25922 and *Psdeudomonas aeruginosa* ATCC 27853 were used 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), treated with RNase, visualized on 7% agarose gel under UV trans-illuminator (Meyers *et al*., 1976) and images stored in 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, purified (Weislander, 1979) and used for transformation.

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 antibiotic-resistant *E. coli* was

added to competent cells of *E. coli* HB101, 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 three different LB agar plates containing ampicillin (100 µg/ml), chloramphenicol (100 µg/ml) and tetracycline (100 µg/ml) and incubated at 37ºC overnight (Sambrook *et al.,* 1989).

RESULTS

In the present study, overall 1210 *E. coli* strains were recovered from different districts and localities of Azad Kashmir, Pakistan. An over whelming majority of *E. coli* isolates (58%) were recovered from children; 57% were from male patients, Majority of the cases were recovered in summer, 441 (36.4%) followed by 435 (35.9%) in autumn, 215 (17.7%) in spring, and 119 (9.8%) in winter. The highest number of stool specimens infected with *E. coli (*40.8%) was recovered in 1998, followed by 36.6% in 1997, 35.0% in 1996 and 33.2% in 1994. The lowest number of strains (21.8%) was isolated in 1995.

Antimicrobial sensitivity

Of 1210 isolates of *E. coli* 48.6% were resistant to carbenicillin (Ca) followed by 47.8% to ampicillin (A), 42.1% to ceftizoxime (CXM), 39.6% to co-trimoxazole (Co), 36.2% to streptomycin (S), 34.0% to amoxicillin (Am), 33.3% to amikacin (Ak), 31.2% to tetracycline (T), 28.5% to erythromycin (Er), 27.9% to nalidixic acid (Na), 25.5% to chloramphenicol (C), 25.4% to gentamicin (G), 21.5% to ceftriaxone (Cz) and 1.2 to ciprofloxacin (CIP). All *E. coli* isolates were sensitive to cefixime (Cfm). It was also observed that the percentage of isolates resistant to any of the antibiotics tested were higher in children than in adults.

Table I shows percentage of isolates resistant to different concentrations of antibiotics. Generally, the isolates showed the highest frequency of resistance against carbenicillin (Ca) at all the four antibiotic concentrations. The lowest frequency of resistance was against ciprofloxacin (CIP) at all the four levels of antibiotics screened. At 100 µg/ml concentration, the isolates showed considerable decrease in the resistance to almost all the antibiotics tested.

Multiple drug resistance was observed in this study ranging from three to ten drugs. Out of 1210 isolates screened for antibiotic resistance, 49% were resistant to three or more antibiotics at 25µg/ml, 46% at 50µg/ml, 23% at 100µg/ml and 10% at 300µg/ml. The resistant isolates showed different patterns of antibiotics resistance. The most common pattern was Ca, A, CXM (Table II).

Plasmid DNA in different strains of E. coli

Of the 593 *E. coli* strains, the plasmids were observed in 185 (31.2%). These were found resistant to three or more antibiotics. The number of plasmids varied from one to five. 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. Analysis of plasmid DNA of *E. coli* 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 Kb (66.5%), 2.3 Kb (58.4%), >9.4 Kb (31.9%), 9.4 Kb (31.9%), 4.3 Kb (29.7%), 6.5 Kb (28.1%) and >6.5 Kb (22.5%). Other plasmids were observed in lesser frequency. The frequency of 2.0 Kb plasmid was 20.5%, and for <23.1 Kb it was 17.8%.

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 185 strains. Thirty eight strains (20.5%) had pattern P1 (4 plasmids), while 33 strains (17.8%) had pattern P2 (3 plasmids), 29 strains (15.7%) had pattern P3 (1 plasmid), while 26 strains (14.0%) had pattern P4 (5 plasmids), 18 strains (9.7%) had P5 (2 plasmid), as 15 (8.1%) had P6 (3 plasmids), 11 (5.9%) had P7 (3 plasmids), 9 (4.9%) had P8 (2 plasmids) and the remaining 6 strains (3.2%) had pattern P9 (2 plasmids).

Table I.- Antibiotic resistance of 1210 *E. coli* isolates at four different concentrations of antibiotics.

Antibiotics	No. of resistant isolates at			
	25 µg/ml	50 µg/ml	100 µg/ml	300 µg/ml
Amikacin (Ak)	403 (33.3%)	361 (29.8%)	208 (17.2%)	47 (3.9%)
Ampicillin (A)	579 (47.8%)	529 (43.7%)	307 (25.4%)	112 (9.2%)
Amoxicillin (Am)	412 (34.0%)	365 (30.2%)	232 (19.2%)	54 (4.5%)
Carbenicillin (Ca)	588 (48.6%)	551 (45.5%)	336 (27.8%)	129 (10.7%)
Cefixime (Cef)	00 (0.0%)	00 (0.0%)	00 (0.0%)	00 (0.0%)
Ceftizoxime (CXM)	510 (42.1%)	468 (38.7%)	283 (23.4%)	95 (7.8%)
Ceftriaxone (Cz)	260 (21.5%)	221 (18.3%)	56 (4.6%)	00 (0.0%)
Chloramphenicol (C)	309 (25.5%)	271 (22.4%)	144 (11.9%)	23 (1.9%)
Ciprofloxacin (Cip)	15 (1.2%)	00 (0.0%)	00 (0.0%)	00 (0.0%)
Co-trimoxazole (Co)	479 (39.6%)	417 (34.5%)	257 (21.2%)	77(6.4%)
Erythromycin(Er)	345 (28.5%)	309 (25.5%)	167 (13.8%)	32 (2.6%)
Gentamicin (G)	307 (25.4%)	256 (21.1%)	123 (10.2%)	16 (1.3%)
Nalidixic acid (Na)	338 (27.9%)	285 (23.5%)	156 (12.9%)	27 (2.2%)
Streptomycin (S)	438 (36.2%)	395 (32.6%)	248 (20.5%)	70 (5.8%)
Tetracycline (T)	378 (31.2%)	337 (27.8%)	202 (16.7%)	38 (3.1%)

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

Antibiotics resistance patterns*	% of resistant isolates at (µg/ml)			
	25	50	100	300
Ca, A, CXM	49	46	23	10
Ca, A, Co	45	42	21	8
Ca, CXM, Co	42	39	19	5
Ca, A, CXM, Co	41	37	16	4
Ca, A, Co, S	36	33	13	3
Ca, CXM, Co, S	30	27	11	3
Ca, A, CXM, Co, Am	22	17	6	2
Ca, CXM, Co, S, Am	15	12	4	1
Ca, A, CXM, S, Am, Ak	13	9	3	1
Ca, A, CXM, Co, Am, T	11	8	2	1
Ca, A, Co, S, Ak, T, Er	9	6	2	1
Ca, CXM, Co, Am, T, Er, Na	7	5	1	-
Ca, A, Co, CXM, T, Na, C	4	3	1	-
Ca, A, CXM, Co, T, Er, Na, C, G, Cz	1	1	-	-
Ca, A, Co, T, Am, Er, Na, G, Cz, Cip	1	1	-	-

*AK, Amikacin; A, Ampicillin; Am, Amoxicillin; Ca, Carbenicillin; Cef, Cefixime; CXM, Ceftizoxime; CZ, Ceftriaxone; C, Chloramphenicol; CIP, Ciprofloxacin; Co, Co-trimoxazole; Er, Erythromycin; G, Gentamicin; Na, Nalidixic acid; S, Streptomycin; T, Tetracycline.

Location of antibiotic resistance gene on plasmid

Of the 185 *E. coli* strains, the plasmids of 45 strains were processed for transformation of *E. coli* HB101 separately for ampicillin (100 µg/ml), chloramphenicol (100 µg/ml) and tetracycline (T-100 µg/ml), plasmids of 34 strains (75.5%) for only ampicillin, 27 (60.0%) for chloramphenicol and 23 (51.1%) for tetracycline resistance. Of the 45 transformations, 38 (84.4%) were successfully

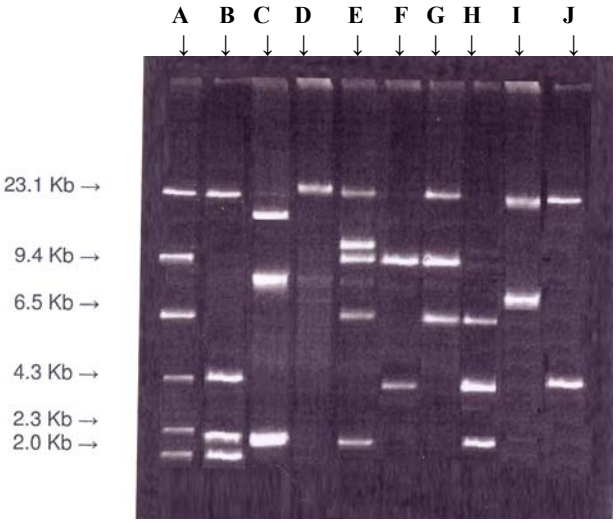

Fig. 1. Plasmid profile (P1-P9) of representative *E. coli* strains isolated from fecal samples of patients with gastroenteritis in Azad Kashmir. Lane A, marker λ DNA cut with Hind III; Lane B, BEc-29; Lane C, BEc-34; Lane D, BEc-128; Lane E, BEc-168; Lane F, BEc-912; Lane G, BEc-945; Lane H, BEc-1476; Lane I, BEc-2516, and Lane J, BEc-2681).

Table III.- Plasmid profile of 185 isolates of *Escherichia coli.*

No. of strains	Presence of plasmid with Molecular weight (Kb) of									Plasmid pattern
	23.1	<23.1	>9.4	9.4	>6.5	6.5	4.3	2.3	2.0	
38	+	-	-	-	-	-	+	+	+	P1
33	-	+	-	-	+	-	-	+	-	P2
29	+	-	-	-	-	-	-	-	-	P3
26	+	-	+	+	-	+	-	+	-	P4
18	-	-	-	+	-	-	+	-	-	P5
15	+	-	-	+	-	+	-	-	-	P6
11	-	-	-	-	-	+	+	+	-	P7
9	+	-	-	-	+	+	-	-	-	P8
6	+	-	-	-	-	-	+	-	-	P9

Table IV.. Transformation of *E. coli* HB101 with plasmids of *E. coli*.

Sample no.	No. of plasmids	Molecular weight of plasmids which were individually transferred to *E. coli* HB101	Transformed plasmids that conferred antibiotic resistance
29	4	23.1Kb, 4.3Kb, 2.3Kb, 2.0Kb.	23.1Kb
945	3	23.1Kb, 9.4Kb, 6.5Kb.	23.1Kb
2516	2	23.1Kb, >6.5Kb.	23.1Kb

accomplished as *E. coli* HB101 acquired antibiotic resistance to ampicillin, chloramphenicol and tetracycline. Plasmids of three strains (BEc-34, BEc-168 and BEc-1476) were successfully transferred to *E. coli* Hb101 shown by the acquisition of resistance to ampicillin, and plasmids of another three strains (BEc-128, BEc-912 and BEc-2681) with chloramphenicol resistance were also successfully introduced into *E. coli* HB101. Plasmids of 34 strains resistant to ampicillin, 27 strains resistant to chloramphenicol, and 23 strains resistant to tetracycline were also successfully introduced into *E. coli* HB101.

In some multiple plasmid strains (BEc-29, BEc-945 and BEc-2516), 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 of 23.1 Kb could only confer ampicillin, chloramphenicol and tetracycline resistance to the competent cells of *E. coli* HB101 (Table IV).

DISCUSSION

E. coli is most frequently isolated from different clinical cases of diarrhoea and others (Tobih *et al.,* 2006). In this study, an overwhelming majority of *E. coli* (58%) were recovered from children, 57% were from male patients. Comparable data was reported in north India by Taneja *et al*. (2004), where 52% patients were children and 70% were below the age of 5 years, where as 73% patients were male. The incidence of infectious diarrhoea in endemic areas usually peaks during the hot, humid, and rainy season. Our study included the months of June to September, which have the same climatic conditions, verifying the high incidence of *E. coli* infections. In this study, 36.4% of *E. coli* were recovered in summer followed by 35.9% in autumn, 17.7% in spring and the lowest number was 9.8% in winter. This was seen in epidemics in most of the other countries, although the seasonality was less pronounced in Africa (Paton *et al.,* 1991). The higher number of cases of diarrhoea investigated during 1998 (23.4%) compared to that in 1994-1997.

Diarrhoea caused by multidrug-resistant bacteria has been recognized as an important public health problem among children in developing countries and is a research priority of the diarrhoeal disease control program of the World Health Organization. *E. coli* has widely been implicated in

various clinical infections as hospital acquired and community infections as reported by Shah *et al.* (2002). Pathogenic isolates of *E. coli* have relatively high potentials for developing resistance (Karlowsky *et al.,* 2004). High frequencies of antimicrobial resistance have been found in enterobacteria, in fecal flora as well as in clinical isolates. *E. coli* isolates from numerous environments have been studied. *E. coli* are the most common cause of Gram negative bacillus infections and have a relatively large potential for developing resistance. Indeed, antimicrobial resistance to β-lactams and other antibiotics has been reported from many countries (Fluit *et al*., 2000). In the present study, clinical isolates of *E. coli* were screened for commonly used antibiotics resistance. *E. coli* accounted for 34.3% of all enteric pathogens isolated and was the second most frequently detected enteropathogen. In this study, overall 48.6% isolates were resistant to carbenicillin followed by 47.8% to ampicillin, 42.1% to ceftizoxime, 39.6% to co-trimoxazole, 36.2% to streptomycin, 34.0% to amoxicillin, 33.3% to amikacin, 31.2% to tetracycline, 28.5% to erythromycin, 27.9% to nalidixic acid, 25.5% to chloramphenicol, 25.4% to gentamicin, 21.5% to ceftriaxone and 1.2 to ciprofloxacin. This data is in consistent with a previous study (Olowe *et al*., 2008), where the prevalence of strains resistance to antimicrobials were; Tetracycline (91.6%), Ampicillin (86.7%), Sulphonamide (77.8%), Gentamicin (39.3%) and Nalidixic acid (4.1%). In addition this finding is in agreement with previous reports in diarrheic patients (Putnam *et al.,* 2005), where 13.3% *E. coli* strains were resistant to ciprofloxacin. Other related cases of drug resistance pattern in blood, high vaginal swab, and diarrhoea have been reported previously by other workers (Tobih *et al.,* 2006; Olowe *et al.,* 2003). In addition, these results are similar with the results of previous study reported by Aibinu *et al.* (2004) where they observed 100% resistance of their *E. coli* isolates to ampicillin and amoxicillin. Multiple antibiotics resistance to useful classes of antibiotics including beta lactams, aminoglycosides and quinolones has generally increased among a number of Gram-negative hospital pathogens. The driving force of the antibiotic resistance being the widespread use of antimicrobial drugs as indicated from the ampicillin usage. In this study our data is in harmony with what was observed by Lamikanra and Okeke (1997). These data confirm that indiscriminate use of antibiotics in this region and along with poor hygiene and infection control (risk factors for antibiotic resistance in bacteria), are highly prevalent in Pakistan and other developing countries (Hart and Kariuki, 1998; Okeke *et al*., 1999).

All *E. coli* isolates were sensitive to cefixime. It was also observed that the percentage of isolates, resistant to any of the antibiotics tested; amikacin (33.6%), amoxicillin (34.2%), chloramphenicol (25.6%) and ciprofloxacin (1.1%) were higher in children than in adults. Resistance to fluoroquinolones has been emerging in recent years, even in countries where antimicrobial resistance rates are low, and multidrug resistance has been reported (Sahm *et al*., 2001). Careful monitoring of the emerging antimicrobial resistance among *E. coli* strains is needed to highlight potential and future problems and may help to formulate intervention strategies.

In the current study, the MICs of fifteen antibiotics against *E. coli* were observed in a comparative account of the antibiotics resistance of isolates at four concentrations 25µg/ml, 50µg/ml, 100µg/ml and 300µg/ml. Generally, the isolates showed the highest frequency of resistance against carbenicillin at all concentrations. The lowest frequency of resistance was against ciprofloxacin at all concentrations. At 100 µg/ml the isolates showed a considerable decrease in the resistance frequency of almost all the antibiotics tested. In this study, the multiple drug resistance was observed from three to ten drugs, and 49% were resistant to three or more antibiotics at 25µg/ml, 46% were resistant to three or more antibiotics at 50µg/ml, 23% were resistant to three or more antibiotics at 100µg/ml and 10% were resistant to three or more antibiotics at 300µg/ml. The resistance to doses as high as 300µg/ml is alarming, because if *E. coli* become resistant to such high levels of antibiotics disease treatment with antibiotics would become quite difficult.

The resistant isolates showed different patterns of antibiotics resistance. The most common pattern was CaACXM at all concentrations. This

data is in agreement with a previous study (Olowe *et al*., 2008), where over 64% of the isolates showed multi-drug resistance. These findings were also in accordance with Laz *et al.* (2001) who reported multidrug resistant *E. coli* resistant to at least eight commonly used antibiotics including ampicillin, tetracycline and chloramphenicol. Oteo *et al.* (2002) have documented multidrug resistance present in 13.92% of isolates; the most prevalent being resistance to ampicillin, co-trimoxazole and ciprofloxacin, which was detected in 59.36% of multiresistant strains and in 8.22% of strains. Overall, this is almost similar with our data.

The multi drug resistance has serious implications for the empiric therapy of infections caused by *E. coli* and for the possible co-selection of antimicrobial resistance mediated by multi drug resistance plasmids (Sherley *et al.,* 2004). According to Miranda *et al*. (2004) infections caused by *E. coli* have become a significant public health problem world wide with the evolution of multi-resistance antibiotic plasmids genes. In the present study, multiple drug resistant (MDR) strains of *E. coli* spp. were processed for isolation of plasmids. The plasmids were observed in (31.2%) MDR strains of *E. coli* spp. which was found resistant to three or more antibiotics used in this research work. The number of plasmids varied from one to five. 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. These findings were in consistent with the findings of Laz *et al*. (2001) who reported that the strains of *E. coli* harbored plasmid of varying molecular weight and molecular size. Similarly, Presterl *et al.* (2003) reported EAEC in 57 of 150 Gabonese children with diarrhoea. These EAEC displayed increased resistance against standard antibiotics such as ampicillin, tetracycline, and trimethoprim. Other characteristics and virulence markers in this population were the presence of the plasmid pCVD432 and the toxins PET and EAST.

In this study, the analysis of plasmid DNA of *E. coli* revealed that all the strains contained a heterogeneous population of plasmids ranging between >23.1 kb to 2.0 kb. These results are comparable with those of who Olowe *et al*. (2008) who reported plasmid of three sizes ranges < 6 – 25 kb in *E. coli* isolates with high multi-drug resistance. Similarly, Jiang *et al.* (2000) reported that the ETEC isolates contained 2–6 plasmids each ranging from 2.0 to 23.0 kb. Plasmid profile pattern 1 (presence of 3.5- and 5.1-kb plasmids) was the most common pattern in 1992 (72%) and 1993 (57%), whereas pattern 2 (with 23.18- and 9.4-kb plasmids) was the most common in 1994 (48%). In study years 1996 and 1997, pattern 6 (22-and 18.1-kb plasmids) was the most frequently identified (42% and 50%, respectively). But our results contradict with the results of Laz *et al*. (2001), where they investigated that each of the twenty drug resistant *E. coli* harbored single plasmid. In addition the current observation is also in agreement with a previous study (Yah *et al*., 2006), where most of the *E. coli* strains screened had one or more resistant R-plasmids. Moreover, 52% of the *E. coli* harbor ampicillin resistant plasmids, the gene responsible for plasmids mediated resistance ampr was found in plasmids varying in size from ≤0.451kb to ≥1.254kb in clinical isolates of *E. coli* strains. The fact that ampr are plasmids borne is supported by plasmids pMG252, the first plasmids found to carry AMPC – type-beta lactamase FOX-5 which have found to encode qnr (quinolones) resistance (Wang *et al*., 2004).

The current study also revealed that there is no consistent relationship between *E. coli* multiple resistant pattern and the number of plasmid bands present. Depending on the number of plasmids, individual strains were grouped into nine different plasmid patterns and were found among (MDR) *E. coli* strains. These results are comparable with results of a previous study (Yah *et al*., 2006). They reported that 3 of the isolates were found resistant to more than 5 antibiotics and had plasmids while another only one plasmid. There was also a high degree of plasmids relatedness among the *E. coli* isolates from the various hospitals because of the presence of similar size plasmids. According to Nashwan *et al*. (2005) the transfer of resistance genes between different bacterial species may go unnoticed by traditional infection control and epidemiological methods, thereby undermining hospital infection control polices. Finally the

presence of plasmid DNA in some *E. coli* multiple resistant strains demonstrated that resistance was plasmid mediated and this could have resulted from the abuse/misuse or other selected pressures sufficient for the broad geographic distribution of ampr genetic linkage.

In the current report, the plasmids of (MDR) *E. coli* strains were processed for transformation into *E. coli* HB101 separately for ampicillin (100 µg/ml), chloramphenicol (100 µg/ml) and tetracycline (100 µg/ml). The transformations (84.4%) were successfully accomplished as *E. coli* HB101 acquired antibiotic resistance to ampicillin, chloramphenicol and tetracycline. The 23.1 Kb plasmids could only confer ampicillin, chloramphenicol and tetracycline resistance to the competent cells of *E. coli* HB101. Recently, in a study the isolates of EAEC exhibited increased resistance to standard antibiotics such as ampicillin, trimethoprim, and tetracycline. It has been explained that the increased antibiotic resistance was due to a readiness for transfer of antibiotic resistance via conjugation probably encoded by the pCVD plasmid (Greenberg *et al.,* 2002). Similarly, Wang *et al.* (2003) reported that the quinolone resistance was transferred from all six *qnr*-positive strains. For the three transconjugants from donors 10, 12, and 29, which each had the same-size *qnr*-hybridizing plasmids, the MIC of ciprofloxacin was the same, 1 µg/ml.

We observed rapid increases in the prevalence of resistance in *E. coli* to most of the older, less expensive antimicrobial drugs used in the management of infections in Azad Kashmir Pakistan. Not only are these strains potential causes of infection, but they are also potential reservoirs of resistance genes that could be transferred to pathogens. For this reason, the trends seen with clinical *E. coli* may also occur with other pathogenic organisms. Studies in other developing countries have shown that the trend in enteric pathogens is toward increasing antibiotic resistance (Hoge *et al.,* 1998).

CONCLUSIONS

Our study emphasizes the need to monitor commensal organisms as well as pathogens by susceptibility testing to guide treatment. Control of antibiotic resistance is needed to conserve the usefulness of the remaining drugs. The future usefulness of these drugs will, however, depend on effective interventions to halt the selection and spread of resistance among enteric organisms. Since antimicrobial resistant patterns are constantly evolving, and it is a present global public health problem, there is the necessity for constant antimicrobial sensitivity surveillance. This will help clinicians provide safe and effective empiric therapies.

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