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Plasmid Borne Antibiotic Resistance Factors in Indigenous *Campylobacter* spp. Isolated from Humans in Azad Jammu and Kashmir, Pakistan

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ARS conceived, designed and supervised the work. He also finalized the article after editing. BA designed and performed the experimental work and prepared the first draft of the article. MHA helped in experimental work. FRS helped in analysis of data and finalizing the draft.

Key words
***Campylobacter*, Antibiotic resistance, Plasmid borne resistance, *C. jejuna*, Antibiotic sensitivity test, Plasmid curing, Multiple drug resistance.**

ABSTRACT

The antimicrobial susceptibility patterns of 146 *Campylobacter* spp. isolated from diarrheal patients admitted to hospitals in Azad Kashmir Pakistan were analyzed to determine their changing trends in response to fifteen antibiotics. *Campylobacter* isolates were identified as *C. jejuni* (66.4%) and *C. coli* (33.6%). An over whelming majority of isolates were recovered from children (72%), 52 % were from male patients. The highest number of strains was isolated in summer (30.8 %) followed by in autumn (25.3 %) and in spring (22.6 %). The lowest number of strains was isolated in winter (21.2 %). The isolates showed highest resistance against carbenicillin followed by ampicillin, co-trimoxazole, streptomycin, amoxicillin, amikacin, ceftizoxime, tetracycline, erythromycin and nalidixic acid. The isolates showed least resistance against ceftriaxone followed by chloramphenicol and gentamicin. All *Campylobacter* isolates were sensitive to cefixime and ciprofloxacin. Multiple drug resistance was observed in this study ranging from three to eight drugs. 36 % were resistant to three or more antibiotics at 25µg/ml, 34 % were resistant to three or more antibiotics at 50µg/ml, 18 % were resistant to three or more antibiotics at 100µg/ml and 8 % were resistant to three or more antibiotics at 300µg/ml. The most common pattern of antibiotic resistance was Carbenicillin+ampicillin+co-trimoxazole. The plasmids were observed in 15.1 % MDR strains of *Campylobacter* spp. which were found resistant to three or more antibiotics. All the strains contained a heterogeneous population of plasmids ranging between 23.1 kb to 2.0 kb. grouped into seven different plasmid patterns. The plasmids (23.1 Kb) could only confer tetracycline resistance to the competent cells of drug sensitive and plasmid-less *Campylobacter* strains. A plasmid-borne tet(O) gene were the main resistance mechanisms for tetracycline.

INTRODUCTION

Campylobacter is motile, somewhat curved, rod like Gram-negative bacteria, with either uni-polar or bi-polar flagella. The development of Skirrow's selective medium enabled routine diagnostic microbiology laboratories to isolate *Campylobacter* and to evaluate their clinical role. This brought to light the true dimension of *Campylobacter* as the leading bacterial cause of human enteritis in the world (Friedman *et al*., 2000; Asmat, 2020).

At least a dozen species of *Campylobacter* have been implicated in human disease, with *C. jejuni* and *C. coli* the most common. Infection with a *Campylobacter* species is one of the most common causes of human bacterial gastroenteritis. Diarrhoea, cramps, abdominal pain and fever develop within 2–5 days of pathogenic *Campylobacter* infection, and in most people, illness lasts for 7–10 days. Campylobacteriosis is usually caused by *C. jejuni* normally found in cattle, swine, and birds, where it is non-pathogenic. Disease-causing bacteria generally get into people via contaminated food, often undercooked or poorly handled poultry, although contact with contaminated drinking water, livestock, or household pets can also cause disease (Ryan and Ray, 2004; Hussain *et al*., 2007; Nisar *et al*., 2018). In developed and developing countries, they are more frequent cause of diarrhoea than, for example, food borne *Salmonella*. In developing countries, *Campylobacter* infections in children under the age of two years are especially frequent, sometimes

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resulting in death (WHO, 2000; Sadiq *et al*., 2019).

However, screening for *Campylobacter* in acute enteric infections is often not a routine matter due to its relatively recent link to human disease and the complexity of procedures for its isolation and identification. As a result, there is little information available specifically on *Campylobacter* in acute enteric infections or antimicrobial resistance from the developing world (Guerrero, 2001). *C. jejuni* and *C. coli* are recognized as the most common causative agents of bacterial gastroenteritis in the world and infections with these organisms occur more frequently than do infections due to *Salmonella*, *Shigella*, or *E. coli* 0157:H7 (Alfredson and Korolik, 2007). *C. jejuni* is a common cause of entero-invasive diarrhoea in man. The disease is often milder than that caused by *Shigella*. In Europe domestic infections occur in young children, whereas travel acquired infections occur in young adults. Outbreaks of *C. enteritis* have been associated with contaminated water and raw milk. Usually diarrhoea due to *Campylobacter* is self-limiting and does not require therapy unless the individual is immuno-suppressed or the infection is extra-intestinal (Engberg, 2006). When adults with *Campylobacter enteritis* receive antibiotics to which the strain is susceptible, they improve more rapidly and they excrete the organism for a shorter time than if they did not receive antibiotics (David and Taylor, 2003).

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 goal of this study was to determine the prevalence of resistance to commonly used antibiotics among clinical isolates of *Campylobacter* strains obtained from patients suffering from diarrhea in Azad Kashmir, Pakistan. The plasmid content of the MDR isolates was examined, and their number correlated with resistance to antibiotics. The plasmid DNA of multiple drug resistant (MDR) bacterial isolates will be transformed into plasmid-less bacterial strains and also suggest the preventive measures.

MATERIALS AND METHODS

Bacterial strains

Campylobacter 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 loop full of stool samples collected from human sources were directly plated on Skirrow's selective agar plates and incubated for 48 h at 42°C in a microaerophilic atmosphere. Suspect colonies were identified as *Campylobacter* isolates on the basis of morphology, Gram stain, motility, as well as oxidase and catalase tests. *C. jejuni and C. coli* were differentiated on the basis of hippurate and indoxyl acetate hydrolysis. Typically, *C. jejuni* is positive for the two tests, while *C. coli* are positive for indoxyl acetate hydrolysis only (Nachamkin *et al*., 2002). Serotyping was performed by serological tests. Only one strain per sample was kept for further studies. The identification numbers used in this study are our own. Bacterial cultures were maintained in freezing 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 medium was used for the cultivation of bacteria (5% sheep blood was added to the LB medium). 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 *Campylobacter* were performed by antibiotic disc diffusion method (Bauer *et al*., 1966), using filter paper discs. The minimum inhibitory concentrations (MICs: 25µg/ml, 50µg/ml, 100µg/ml, and 300µg/ml) of fifteen commonly used antibiotics were determined by agar dilution method and the MIC was defined as the lowest concentration in which there was no visible growth. Reference strains *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were tested regularly as controls according to the National Committee for Clinical Laboratory Standards (Anonymous, 1993).

Plasmid DNA isolation

Plasmid DNA was isolated from the multiple antibiotics resistant strains according to Birnboim and

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

Plasmid curing

Plasmid curing was performed according to Hirota (1960). Five 100 ml flasks were taken and 20 ml acridine orange broth was poured into each flask, then in each flask inoculums ($2x10^{-2}$ - $5x10^{-2}$ bacteria) was added to varying concentrations (25µg/ml, 50µg/ml, 100µg/ml, 200µg/ml, 400µg/ml) of acridine orange broth and the flasks were placed into the shaking water bath for 24 h at 42°C. Culture containing the highest concentration of acridine orange in which growth was clearly visible was diluted and spread on LB agar plates (5% sheep blood was also added to LB agar) containing appropriate antibiotics by using sterilized glass spreader. The plates were incubated at 42°C for 18-24 h. After incubation the plates were observed in order to note the visible growth of bacteria. A single colony of each bacterial strain was picked up and inoculated in 10 ml of LB broth and the culture of the bacterial strains was grown at 42°C in the shaking water bath for overnight. 50 µl of the overnight bacterial culture was inoculated in 50 ml of LB broth. Overnight culture of bacterial strains was grown at 42°C in the shaking water bath till O.D=1 at 600 nm. Crude lysates prepared from cured *Campylobacter* by the method of Birnboim and Doly (1979), were visualized on agarose gel (Meyers *et al*., 1976).

Transformation

Bacterial transformation was performed by the method of Sambrook *et al*. (1989). All the isolates were tested for the ability to transfer their determinants. *Campylobacter* and *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 *Campylobacter* was added to competent cells of *Campylobacter* and *E. coli* HB101, prepared, incubated on ice for 30 min and then at 42°C for 2 min. One ml of pre-warmed LB broth was then added to this mixture and re-incubated at 42°C at 60 rpm for 80 min. The whole mixture was then spread on two different LB agar plates (5% sheep blood was also added to LB agar) containing ampicillin (100 µg/ml), chloramphenicol (100 µg/ml) and tetracycline (100 µg/ml) and incubated at 42°C overnight.

RESULTS

Infections age-wise, gender-wise and season-wise

A total of 146 fecal specimens infected with *Campylobacter* spp. were analyzed for the 5-year period. Repeat fecal specimens from the same patients were excluded. Of the 146 fecal specimens infected with *Campylobacter* spp. an overwhelming majority, 105 (72 %) were from children, while 41 (28 %) were from adults. Of the 146 fecal specimens infected with *Campylobacter* spp., 76 (52 %) were from male patients and 70 (48 %) from females. Forty five (30.8 %) cases were referred in summer followed by 37 (25.3 %) in autumn, 33 (22.6 %) in spring and 31 (21.2 %) in winter (Table I).

The isolation rate of *Campylobacter* in diarrheic children (5 years of age) passing blood and mucus in their stools was 105 (72 %). No child was infected with more than one enteropathogen. Overall the highest proportion of stool specimens infected with enteropathogen were in the age group 36-47 months (7.6 %), followed by 6.5 % in 24-35 months, 5.7 % in 12-23 and 48-60 months. The lowest infestation was observed in the age groups 0-11 months (3.0 %). *Campylobacter* was less frequently isolated in children less than one year of age (25 of 105) compared with older age groups (80 of 105) (Table II).

Table I.- Age-wise, gender-wise and season-wise distribution of *Campylobacter* isolated from fecal specimens of patients with gastroenteritis in Azad Kashmir, Pakistan.

Fecal specimens with bacterial strains	Age-wise incidence		Gender-wise incidence		Season-wise incidence*			
	Children	Adults	Male	Female	Summer	Autumn	Spring	Winter
Campylobacter (n=146)	105 (72%)	41 (28%)	76 (52%)	70 (48%)	45 (3.6%)	37 (3.6%)	33 (4.8%)	31 (5.6%)
Total (n=3529)	2125 (60%)	1404 (40%)	2054 (58%)	1475 (42%)	1256	1031	692	550

*The percentage in season-wise incidence has been calculated with reference to total number of organism in a particular season.

Table II.- Occurrence of *Campylobacter* in bloody diarrhoea from children (n=105) according to different age groups in Azad Kashmir, Pakistan.

Age (months)	Total No. of pathogens (%)	*Campylobacter*	
		No.	%
0-11	824 (38.8)	25	3.0
12-23	644 (30.3)	37	5.7
24-35	246 (11.6)	16	6.5
36-47	184 (8.6)	14	7.6
48-60	227 (10.7)	13	5.7
Total	2125	105	4.9

Table III.- Yearly distribution and percentages of *Campylobacter* from fecal specimens of patients with gastroenteritis in Azad Kashmir, Pakistan.

Year	Total No. of fecal specimens	No. of specimens with *Campylobacter* (%)
1	807 (22.9 %)	33(4.1%)
2	523 (14.8 %)	26(4.9%)
3	771 (21.8 %)	28(3.6%)
4	603 (17.1 %)	29(4.8%)
5	825 (23.4 %)	30(3.6%)
Total	3529	146(4.1%)

Infections year-wise

During first year of investigation, 33 (4.1 %) fecal specimens infected with *Campylobacter* spp. were referred to the laboratory, whereas this number was 26 (4.9 %), 28 (3.6 %), 29 (4.8 %) and 30 (3.6 %) in the subsequent years (Table III).

Antimicrobial sensitivity of Campylobacter *spp.*

One hundred and forty six clinical isolates of *Campylobacter* spp. were identified. The antibiotic resistance of *Campylobacter* spp. strains was checked with paper disc diffusion method. After 24 h of incubation the following results were obtained (Table IV).

Susceptibility profiles of both *C. jejuni* and *C. coli* were almost similar during the study period. Overall 37.7% isolates were resistant to carbenicillin, 36.3% to ampicillin, 28.1% to co-trimoxazole, 24.6% to streptomycin, 23.3% to amoxicillin, 22.6% to amikacin, 20.5% to ceftizoxime, 19.8% to tetracycline, 17.1% to erythromycin, 16.4% to nalidixic acid, 14.4% to gentamicin, 13.0% to chloramphenicol and 10.3% to ceftriaxone. All *Campylobacter* isolates were sensitive to cefixime and ciprofloxacin. Table IV shows that resistance of isolates to amikacin (22.8%), amoxicillin (23.8%), ceftizoxime (20.9%), ceftriaxone (10.5%), chloramphenicol (13.3%), co-trimoxazole (28.6%), streptomycin (24.8%) and tetracycline (20.0%) was higher in children than in adults.

The MICs of twenty antibiotics against one hundred and forty six isolates of *Campylobacter* are shown in a comparative account of the antibiotics resistance of isolates at four levels 25µg/ml, 50µg/ml, 100µg/ml and 300µg/ml (Table V). Generally, the isolates showed the highest resistance against carbenicillin at all the four doses. The lowest resistance was recorded against chloramphenicol and ceftriaxone at all the four doses of antibiotics. At 100µg/ml level the isolates showed a considerable decrease in the antibiotic. The isolates were highly sensitive to cefixime and ciprofloxacin.

Table IV.- Antibiotics resistance pattern of the 146 *Campylobacter* isolated from children and adults.

Antibiotics (25µg/ml)	No. (%) of resistant isolates		
	Children (n=105)	Adults (n=41)	Total (n=146)
Amikacin	24 (22.8 %)	9 (21.9 %)	33 (22.6 %)
Ampicillin	38 (36.1 %)	15 (36.6 %)	53 (36.3 %)
Amoxicillin	25 (23.8 %)	9 (21.9 %)	34 (23.3 %)
Carbenicillin	39 (37.1 %)	16 (39.0 %)	55 (37.7 %)
Cefixime	00 (0.0 %)	00 (0.0 %)	00 (0.0 %)
Ceftizoxime	22 (20.9 %)	8 (19.5 %)	30 (20.5 %)
Ceftriaxone	11 (10.5 %)	4 (9.7 %)	15 (10.3 %)
Chloramphenicol	14 (13.3 %)	5 (12.2 %)	19 (13.0 %)
Ciprofloxacin	00 (0.0 %)	00 (0.0 %)	00 (0.0 %)
Co-trimoxazole	30 (28.6 %)	11 (26.8 %)	41 (28.1 %)
Erythromycin	18 (17.1 %)	7 (17.1 %)	25 (17.1 %)
Gentamicin	15 (14.3 %)	6 (14.6 %)	21 (14.4 %)
Nalidixic acid	17 (16.2 %)	7 (17.1 %)	24 (16.4 %)
Streptomycin	26 (24.8 %)	10 (24.4 %)	36 (24.6 %)
Tetracycline	21 (20.0 %)	8 (19.5 %)	29 (19.8 %)

Multiple drug resistance was observed in this study ranging from three to eight drugs. Out of one hundred and forty six isolates, screened for antibiotic resistance, 36 % were resistant to three or more antibiotics at 25µg/ml, 34 % were resistant to three or more antibiotics at 50µg/ml, 18 % 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 CaACo. In addition, 1% of strains were found to be resistant to eight antibiotics (Ca, A, Co, S, T, Er, Na and G) at 50µg/ml (Table VI).

Table V.- Occurrence of antibiotic resistance of 146 *Campylobacter* isolates at four different concentrations.

Antibiotics	No. of resistant isolates			
	25 µg/ml	50 µg/ml	100 µg/ml	300 µg/ml
Amikacin	33 (22.6 %)	28 (19.2%)	13 (8.9%)	6 (4.1%)
Ampicillin	53 (36.3 %)	47 (32.2%)	24 (16.4%)	12 (8.2%)
Amoxicillin	34 (23.3 %)	29 (19.9%)	14 (9.6%)	7 (4.8%)
Carbenicillin	55 (37.7 %)	50 (34.2%)	25 (17.1%)	13 (8.9%)
Cefixime	00 (0.0 %)	00 (0.0%)	00 (0.0%)	00 (0.0%)
Ceftizoxime	30 (20.5 %)	25 (17.1%)	12 (8.2%)	5 (3.4%)
Ceftriaxone	15 (10.3 %)	11 (7.5%)	5 (3.4%)	00 (0.0%)
Chloramphenicol	19 (13.0 %)	14 (9.6%)	7 (4.8%)	1 (0.7%)
Ciprofloxacin	00 (0.0 %)	00 (0.0%)	00 (0.0%)	00 (0.0%)
Co-trimoxazole	41 (28.1 %)	37 (25.3%)	18 (12.3%)	9 (6.2%)
Erythromycin	25 (17.1 %)	20 (13.7%)	10 (6.8%)	4 (2.7%)
Gentamicin	21 (14.4 %)	17(11.6%)	8 (5.5%)	3 (2.0%)
Nalidixic acid	24 (16.4 %)	19 (13.0%)	9 (6.2%)	3 (2.0%)
Streptomycin	36 (24.6 %)	30 (20.5%)	15 (10.3%)	8 (5.5%)
Tetracycline	29 (19.8 %)	24 (16.4%)	11 (7.5%)	5 (3.4%)

Table VI.- Multiple antibiotic resistance patterns occurring in *Campylobacter* isolated from various clinical sources of Azad Kashmir, Pakistan.

Antibiotic resistance patterns	Percentage of resistant isolates			
	25 µg/ml	50 µg/ml	100 µg/ml	300 µg/ml
Ca, A, Co	36	34	18	8
Ca, A, S	34	32	16	7
Ca, A, S, Am	32	30	15	6
Ca, Co, S, Ak	25	23	12	5
Ca, A, Co, Am	21	19	9	3
Ca, A, Co, S, Am	14	12	6	2
Ca, A, Co, S, Ak	10	8	4	1
Ca, A, Co, S, Am, CXM	7	5	2	1
Ca, A, Co, S, Ak, T, Er	5	3	1	1
Ca, Co, Am, Ak, T, Er, Na	3	2	1	-
Ca, A, Co, CXM, T, Na, C	1	1	-	-
Ca, A, Co, S, T, Er, Na, G	1	1	-	-

Plasmid

A total of 1744 multiple antibiotics resistant isolates out of 3529 were selected for the plasmid DNA screening. Fifty three isolates of *Campylobacter* spp. were found resistant to three or more antibiotics. Of these 53 strains, the plasmids were observed in 8 (15.1%) antibiotics resistant strains. These were found resistant to three or more antibiotics used in this work. The number of plasmids varied from one to three. The plasmid pattern was determined by the presence or absence of a single plasmid within a group of strains.

Analysis of plasmid DNA of *Campylobacter* revealed that all the strains contained a heterogeneous population of plasmids ranging between 23.1kb to 2.0kb. The molecular size of all plasmids was determined by comparison with a bacteriophage lambda DNA digest with *Hind* III (Fig. 1).

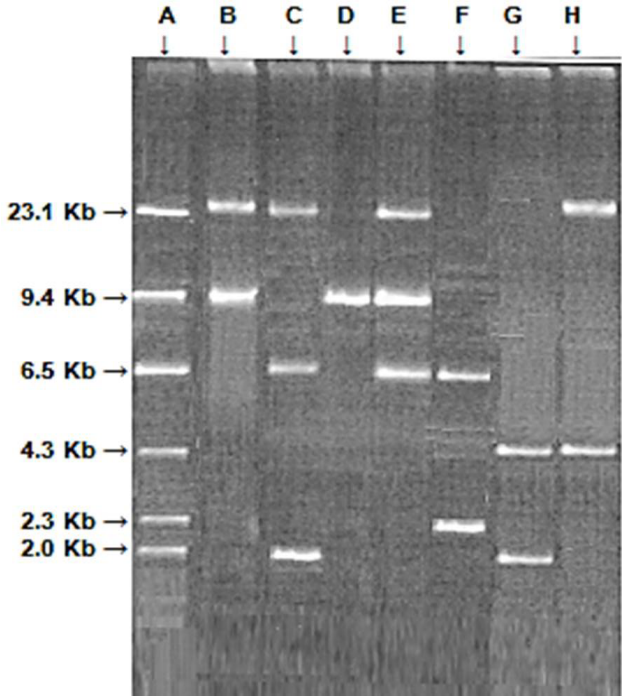

Fig. 1. Hetrogenous populaion of plasmids (23.1kb to 2.0kb) in different *Campylobacter* isolates from various clinical sources of Azad Kashmir, Pakistan (Lanes B-H). Lane A shows bacteriophage λ DNA digested with *Hin*d III.

The most dominant plasmids were 23.1Kb, 9.4Kb, 6.5Kb, 4.3Kb and 2.0Kb. The frequency with which they were encountered was 62.5%, 50.0%, 37.5%, 25.0% and 25.0%, respectively. Other plasmids were observed in lesser frequency. The frequency of 2.3Kb plasmids was 12.5%.

Based on molecular weight, the pattern of different plasmids was diverse. Depending on the number of plasmids, individual strains were grouped into different patterns. Seven different plasmid patterns, designated P1-P7, were found among the 8 strains. Two strains (25.0%) had pattern P1 (2 plasmids), while the remaining six strains each one (12.5%) had its own pattern P2 (3 plasmids), P3 (1 plasmid), P4 (3 plasmids), P5 (2 plasmids), P6 (2 plasmids) and P7 (2 plasmids), respectively (Table VII).

Table VII.- Plasmid profile analysis of eight isolates of *Campylobacter.*

No. of strains	Plasmids (Kb)						Plasmid pattern
	23.1	9.4	6.5	4.3	2.3	2.0	
2	+	+	-	-	-	-	P1
1	+	-	+	-	-	+	P2
1	-	+	-	-	-	-	P3
1	+	+	+	-	-	-	P4
1	-	-	+	-	+	-	P5
1	-	-	-	+	-	+	P6
1	+	-	-	+	-	-	P7

Table VIII.- Effect of acridine orange mediated plasmid curing on the antibiotic resistance pattern of *Campylobacter* isolates.

Isolate No.	Resistance pattern	
	Pre-curing	Post-curing
BC-2217	CaACo	Co
BC-2241	CaACoSTErNaG	CoErNaG

Location of antibiotic resistance gene

For determination of location of antibiotic resistance gene two approaches were adopted (i) plasmid curing, which would obliterate the antibiotic resistance if the gene were located on the plasmid, and (ii) its restoration after transformation of a suitable host with antibiotic resistant gene. These approaches should provide evidence for location of antibiotic gene on the plasmid.

Plasmid curing

The representative multiple drug resistant isolates *Campylobacter* (BC-2217 and BC-2241) were selected for plasmid curing. Acridine orange was used as curing agent during this study for the elimination of plasmid. In *Campylobacter* (BC-2217 and BC=2241) a discrete plasmid band was observed, which was absent from the same *Campylobacter* isolate, cured of their plasmid by treatment with acridine orange. Out of 100 colonies each from two treated cultures some had lost the resistance to one or the other antibiotic. Effects of plasmid curing on the drug resistance determinants of *Campylobacter* isolates are shown in Table VIII.

Transformation

Of the 8 *Campylobacter* tetracycline-resistant isolates, the plasmids were processed for transformation of a drug sensitive and plasmid-less *Campylobacter* (BC-2285) strains and E. coli HB101 separately for tetracycline resistance (MIC-100 µg/ml). Of the 8 transformations, 5 (62.5%) were successfully accomplished as *Campylobacter* acquired antibiotic resistance to tetracycline. *E. coli* HB101, however, could not be successfully transformed with the same plasmids of 8 strains resistant to tetracycline.

In some multiple plasmid strains (BC-2217, BC-2241 and BC-2287), all the DNA bands of different molecular sizes were cut out of the gel, extracted, purified and then successfully transferred to a drug sensitive and plasmid-less *Campylobacter* (BC-2285) individually. Tetracycline resistance is most likely due to the transfer plasmids 23.1Kb carrying the tet (O) gene between isolates. The plasmids (23.1 Kb) could only confer tetracycline resistance to the competent cells of a drug sensitive and plasmid-less *Campylobacter* (BC-2285) (Table IX).

Table IX.- Transformation of a drug sensitive and plasmid-less *Campylobacter* with plasmids of *Campylobacter*.

Sample No.	No. of plasmids	Molecular weight*	Transformed plasmids**
2217	2	23.1Kb, 9.4Kb	23.1Kb
2241	3	23.1Kb, 6.5Kb, 2.0Kb	23.1Kb
2287	3	23.1Kb, 9.4Kb, 6.5Kb	23.1Kb

*, molecular weight of plasmids which were individually transferred to a drug sensitive and plasmid-less *Campylobacter*; **, transformed plasmids that conferred antibiotic resistance.

DISCUSSION

Pathogenic Campylobacter

Pathogenic bacteria usually invade the small intestine and colon and cause enterocolitis (inflammation of the small intestine and colon). Bacterial enterocolitis is characterized by signs of inflammation (blood or pus in the stool, fever) and abdominal pain and diarrhoea. Classical

food poisoning is caused by a variety of different bacteria. The most common are *Campylobacter*. Sometimes food poisoning is called bacterial gastroenteritis or infectious diarrhoea. These bacteria usually are acquired by drinking contaminated water or eating contaminated foods such as vegetables, poultry, and dairy products.

In the present study, *Campylobacter* spp. were recovered from different districts and localities of Azad Kashmir, Pakistan. In this study, an overwhelming majority of *Campylobacter* (72%) were recovered from children, 52 % 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, whereas 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 whole year duration, which has the same climatic conditions, verifying the high incidence of *Campylobacter* spp. In this study, the highest number of *Campylobacter* was recovered in summer (30.8%) followed by autumn (25.3%), spring (22.6%) and winter (21.2%). 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 were investigated during 1995 (4.9 %) compared with that in 1994 (4.1%) and 1996-1998 (3.6%, 4.8%, 3.6%).

The acute diarrhoea is a major cause of morbidity and mortality in infants and young children globally. Most diarrhoea episodes are self-limited and caused by an infectious agent. However, a microorganism is identified in approximately 50 % of the cases (Tolia, 2002). There are multiple causes of acute diarrhoea in children. In the present study, the isolation rate of *Campylobacter* in diarrhoeic children (5 years of age). The highest proportion of *Campylobacter* spp. was observed in the age group 36-47 months (7.6 %). Comparable results were presented by Khalil *et al*. (1998). In addition, the *Campylobacter* was most commonly identified pathogen in the stools of children less than one year of age. Although bacterial enteric infections are often self-limited, specific antibiotic treatments may shorten the duration of illness in normal hosts and prevent serious complications such as sepsis and protracted diarrhoea in young infants or in children with underlying conditions such as immunosuppression or malnutrition. Enteric bacterial pathogens show increased resistance to standard therapy. Antibiotics are variably (usually minimally) effective; their use may prolong the carrier status. Antimicrobial therapy in the non-immunocompromised host older than newborn age is indicated for infection with *Vibrio cholerae*, *Shigella*, *Clostridium difficile*, and *Giardia lamblia* (Pickering and Cleary, 1998).

Antibiotic resistance

In this study, the antimicrobial susceptibility patterns for *Campylobacter* spp. isolated from diarrhoeal patients admitted to hospitals in the districts of Muzaffarabad, Mirpur, and Rawalakot Azad Kashmir, Pakistan were analyzed to determine their changing trends in response to commonly used fifteen antibiotics. It showed a high prevalence of antimicrobial drug resistance in *Campylobacter* spp. isolates. In this study, the results indicate that antibiotic resistance among indigenous clinical *Campylobacter* spp. is very high against aminoglycosides, beta-lactams (penicillins and cephalosporins), chloramphenicol, macrolides, quinolones, sulfonamides, tetracyclines and trimethoprim. Unfortunately, the options for antibiotic therapy of gastroenteritis have narrowed considerably in recent years as bacterial resistance has increased (Ghosh and Sehgal, 1998; Khan *et al*., 2020).

Campylobacteriosis is a significant public health problem in many developed countries. At least a dozen species of *Campylobacter* have been implicated in human disease. Infection with a *Campylobacter* species is one of the most common causes of human bacterial gastroenteritis. *Campylobacter* species, particularly *C. jejuni* and *C. coli*, are recognized as one of the most frequent causes of acute diarrhoeal disease in humans throughout the world. Campylobacteriosis is a collective description for infectious diseases caused by members of the genus *Campylobacter* (Coker *et al*., 2002). Of the infectious diseases caused by members of the *Campylobacter* genus, *Campylobacter* gastroenteritis due to *C. jejuni* and *C. coli* is the only form of disease that is of major public health importance and increasing antimicrobial resistance in both medicine and agriculture in *Campylobacter* is recognized by various national authorities including the World Health Organization (WHO) as a major emerging public health concern. Although *C. jejuni* and *C. coli* have both been implicated as causes of diarrhoeal disease, including the most common cause of diarrhoea in travelers from developed nations, *C. jejuni* is the species most frequently isolated in cases of human infection (Padungton and Kaneene, 2003). In both industrialized and developing countries, *Campylobacter* remains one of the most common bacterial causes of diarrhoea.

In the present study, clinical isolates were resistant to carbenicillin followed by ampicillin, co-trimoxazole, streptomycin, amoxicillin, amikacin, ceftizoxime, tetracycline, erythromycin and nalidixic acid. The lowest resistance was observed against ceftriaxone followed by chloramphenicol and gentamicin. Almost similar patterns of resistance have been reported in Indonesia from 1995 to 2001 (Tjaniadi *et al*., 2003) and in Thailand (Hoge *et al*., 1998), where many isolates of *C. jejuni* were observed

resistant to ampicillin, trimethoprim/sulfamethoxazole, tetracycline, cephalothin, ceftriaxone and fluoroquinolones. This finding is in agreement with a previous report from Italy (Mazi *et al*., 2008), where many isolates of *C. jejuni* were observed resistant to ciprofloxacin, tetracycline, and erythromycin. Wardak *et al*. (2007) reported that the *C. jejuni* and *C. coli* clinical isolates from Poland, all were susceptible to erythromycin and *C. jejuni* isolates 55.9% and 13.7% were resistant to ciprofloxacin and tetracycline, respectively, which contradict with our findings in the current study. In our finding *Campylobacter* isolates were resistant against the erythromycin (17.1%) and were also resistant against tetracycline (19.8%) where as we observed all *Campylobacter* isolates were susceptible to ciprofloxacin.

Increased antibiotic resistance is being reported in *C. jejuni*, particularly tetracycline and ciprofloxacin resistance (Nachamkin *et al*., 2000). Worldwide, tetracycline resistance (Tcr) frequencies among human isolates of *C. jejuni* are high; for example, 55 to 56% in North America (Gaudreau and Gilbert, 1998) and up to 95% in Thailand (Li *et al*., 1998). In Alberta, Canada, Tcr rates in human clinical isolates of *C. jejuni* were 6.8 and 8.6% in 1980 and 1981, respectively (Taylor *et al*., 1986). Ciprofloxacin resistance frequencies in *C. jejuni* have increased dramatically in the last few decades, approaching 88% in Spain (Ruiz *et al*., 1998). Fortunately, the prevalence of erythromycin resistance has remained low often well below 10% of isolates (Nachamkin *et al*., 2002) However, a recent Canadian study has identified a sudden increase in erythromycin resistance to 12 % (Gaudreau and Gilbert, 2003). Available data on macrolide resistance in percentage among *C. jejuni*, *C. coli* and *C. jejuni/coli* combined, isolated from human sources around the world since 1997. Almost all studies report a higher frequency of erythromycin resistance in *C. coli* than in *C. jejuni* with rates reported in proportions ranging from 0% to 20% in *C. jejuni* and 0% to 29% in *C. coli*. In a number of industrialized countries, a higher proportion of *C. coli*, including macrolide-resistant *C. coli*, have been reported among travel-related patients than among domestically acquired infections. Trend over time for macrolide resistance showed stable low rates in most countries, which is comforting as erythromycin or, alternatively, one of the newer macrolides, such as azythromycin, is the drug of choice for treating *C. jejuni/coli* enteritis. The macrolide resistance mechanism in *Campylobacter* is likely to be chromosomal mutations in the drug sensitive target. Thus, resistance to macrolides in *Campylobacter* will spread with the bacteria and not be transferable to other bacteria. Tetracycline resistance is most likely due to the transfer plasmids carrying the tet (O) gene between isolates. In 1987 and 1990, *Campylobacter* isolated from U.S. troops in Thailand were susceptible to fluoroquinolones but the incidence of *Campylobacter* resistance to fluoroquinolones has risen from 40 % in 1993 to 84 % in 1995 (Hoge *et al*., 1998). In addition, a report from Quebec, Canada indicates that resistance to ciprofloxacin has increased three-fold in the period from 1985 through 1997 (Gaudreau and Gilbert, 1998). Ciprofloxacin resistance in *C. jejuni* in Indonesia increase from 0 % in 1997 to 43 % in 2000 ($P > 0.05$) (Tjaniadi *et al*., 2000). In the current study, all strains of *Campylobacter* were found sensitive to cefixime and ciprofloxacin. This finding contradicts with a previous report from Indonesia (Tjaniadi *et al*., 2003), but is in agreement with the report from Quebec, Canada (Nachamkin *et al*., 2000) and from Thailand (1985–1997), which indicated the resistance to ciprofloxacin among *Campylobacter* strains (Taylor *et al*., 1997). It was also observed that the percentage of isolates, resistant to any of the antibiotics tested; amikacin, amoxicillin, ceftizoxime, ceftriaxone, chloramphenicol, co-trimoxazole, streptomycin and tetracycline were higher in children than in adults.

Of the fifteen antibiotics tested against isolates of *Campylobacter* spp. ceftriaxone and chloramphenicol showed the lowest frequency of resistance. Comparable results were reported by Pratt and Korolik (2005), where the *C. jejuni* and *C. coli* from clinical isolates in Australia were highly resistant to tetracycline. In the current study, the multiple drug resistance (MDR) was observed in *Campylobacter* spp. from three to eight drugs. It was noted that 36 % isolates were resistant to three or more antibiotics at 25µg/ml, 34 % were resistant to three or more antibiotics at 50µg/ml, 18 % were resistant to three or more antibiotics at 100µg/ml and 8 % were resistant to three or more antibiotics at 300µg/ml. The resistance to doses as high as 300µg/ml is alarming, because if *Campylobacter* spp., 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 CaACo at all the four levels.

Antimicrobial resistance has become a major public health concern in both developed and developing countries in recent years (Nachamkin *et al*., 2002). *Campylobacter* with resistance to ciprofloxacin or other fluoroquinolones, macrolides and lincosamides, chloramphenicol, aminoglycosides, tetracycline, ampicillin and other β-lactams, cotrimoxazole, and tylosin have been reported (Moore *et al*., 2006). In the past decade, a rapidly increasing proportion of *Campylobacter* strains world-wide have developed resistance to the fluoroquinolones. In 1995, the incidence of fluoroquinolone resistance in

Campylobacter isolates from Thailand was reported as 84% and, in 1997–1998, the incidence of fluoroquinolone resistance in Spain was reported as 72%. Incidence of resistance to the fluoroquinolones has also increased in the United States, United Kingdom, and the Netherlands. In 1998–1999, the proportion of *Campylobacter* isolates resistant to fluoroquinolones was reported as 10%, 18%, and 29%, respectively (Allos, 2001). The macrolides are now generally considered to be the optimal drug for treatment of *Campylobacter* infections; however, resistance to macrolides in human isolates in some countries is becoming a major public health concern. Although macrolide resistance is infrequent and stable in most countries, resistance rates of *C. jejuni* to the macrolides of 10% and 11% have been reported in Taiwan and Spain, respectively (Gaudreau and Gilbert, 2003), while higher rates of resistance of 31%, 51%, and 79% have been reported in Bulgaria, Singapore, and Nigeria, respectively (Gibreel and Taylor, 2006); resistance to macrolides is found to be more prevalent in *C. coli* than *C. jejuni* (Padungton and Kaneene, 2003). Despite decades of use, however, trends over time for erythromycin resistance of human *Campylobacter* isolates show stable and low rates in countries including Sweden, Finland, and Japan. The use of antimicrobial agents, including macrolide derivatives in food animals, creates selective pressure for the emergence and dissemination of resistance among human pathogens that have food-animal reservoirs (Gibreel and Taylor, 2006). The emergence of *Campylobacter* isolates resistant to both quinolones and azithromycin in Thailand has been reported (Hoge *et al*., 1998) and threatens their usefulness in this demographic region.

According to the results reported here, it is possible to conclude that health workers are not aware of the worldwide hazard of bacterial resistance as denounced by the World Health Organization (2000). This situation further reinforces the urgent need for establishing a rational, organized control program for antimicrobial usage in human and animal health.

Location of antibiotic resistance gene

The drug resistance in bacterial population is attributable to genetic mechanisms. Regarding genetic mechanism most drug resistant microbes emerged as a result of genetic changes and subsequent processes by antimicrobial drugs. The drug resistance may be chromosomal DNA or plasmid DNA mediated. The plasmid mediated drug resistance is caused due to the presence of drug resistant gene(s) harboring on the plasmid DNA. These gene(s) confer the drug resistance phenomenon in the host organism (Meyers *et al*., 1976). Plasmids carrying drug resistance phenotype are known as R-factor which is responsible for the spread of multiple drug resistance among bacteria. R-factor consists of two components *i.e.* resistance transfer factor (RTF) and resistance determinant 'r'. The complete plasmid (RTF+r) is called R-factor (Patwary, 1994). Plasmid-specified resistance to antimicrobial agents has become the widespread mechanism of bacterial resistance to antibiotics. 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). 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 (Tjaniadi *et al*., 2003).

In the present study, MDR isolates of *Campylobacter* spp. were found resistant to three or more antibiotics. The plasmids allow the movement of genetic material, including antimicrobial resistance genes between bacterial species and genera (Miranda *et al*., 2004). Unfortunately, there are still large gaps in our understanding of how new multi-resistance plasmids evolve. The analysis of the bacterial collections from the pre-antibiotic era indicates that although plasmids were present in some of the strains but did not harbor antibiotic resistance genes (Chakrabarty *et al*., 1990).

A number of antibiotic resistance mechanisms are present in *C. jejuni*. Tcr is primarily mediated by a plasmid-encoded *tet* (O) gene (Taylor *et al*., 1987). Tet (O), a ribosomal protection protein, confers resistance by displacing tetracycline from its primary binding site on the ribosome (Connell *et al*., 2003). Previous studies have determined that the *tet* (O) gene in *C. jejuni* mediates MICs of up to 128 µg of tetracycline/ml. Kanamycin resistance (Kmr) in *C. jejuni* is most frequently associated with the existence of the *aphA-3* gene which is identified in most cases on large plasmids in the range of 40 to 130 kb. Resistance to erythromycin is most likely due to an alteration of the target site on the 23S rRNA of the *C. jejuni* ribosome. Ciprofloxacin resistance depends on mutations within the *gyrA* gene, which encodes the A subunit of the DNA gyrase enzyme. A single point mutation at Thr-86, Asp-90, or Ala-70 in *gyrA* can result in fluoroquinolone

resistance (Trieber and Taylor, 2000).

In the present report, plasmids were observed in 15.1 % MDR strains of *Campylobacter* spp. which is comparable with the results of study by Lee *et al*. (1994), where plasmid content in human isolates of *C. jejuni* were reported to vary from 13 to 52%, with the majority being resistance plasmids. In our study, the number of plasmids varied from one to three. This finding is in agreement with a previous report from Italy[26] where many isolates of *C. jejuni,* with the exception of a tetracycline sensitive human isolate, harbored plasmids ranging in size from 15 kb to 35 kb. The commonest plasmids were 23 kb and 35 kb long and all isolates with more than one plasmid carried the 23 kb plasmid. In current study, individual strains were grouped into seven different plasmid patterns. Wardak *et al*. (2007) reported almost similar results in which, eight different profiles of plasmids were noted, indicating heterogeneity of these plasmids. Nevertheless, five *C. jejuni* plasmids and one *C. coli* plasmid revealed the same predominating profile. This suggested that these isolates harbored the same horizontally transferred plasmid.

Location of antimicrobial resistance determinants on plasmid

Plasmid curing

The location (chromosomal or extra chromosomal) of drug resistance determinants was also confirmed by plasmid curing strategies. *Campylobacter* (BC-2217 and BC-2241) lost their plasmids after treatment with acridine orange. Resultantly, some of the resistance markers were stably lost (excluding co-trimaxazole, erythromycin, nalidixic acid and gentamicin in terms of the MDR *Campylobacter* strains; thereby showing the chromosomal location of these two markers). However, a total loss to ampicillin and carbenicillin was found in the cultures. Similar studies were performed by earlier workers (Joan, 1976; 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).

Transformation

In this study, 62.5% transformations of drug sensitive and plasmid-less *Campylobacter* strains and *E. coli* HB101were successfully accomplished. These results were comparable with the results of a previous study reported by Batchelor *et al*. (2004) where the conjugative transfer of the Tcr plasmids has been demonstrated between *Campylobacter* spp. however, conjugative transfer to *E. coli* was not possible, suggesting that the host range was restricted to *Campylobacter* spp. Padungton and Kaneene (2003) have reported *that i*n both *C. jejuni* and *C. coli*, resistance to tetracycline was found to be located on a self-transmissible plasmid encoding an RPP gene, designated tet(O). Plasmid content in human isolates of *C. jejuni* has been reported to be between 13% and 52%, with the majority being resistance plasmids. Kanamycin-resistance phosphotransferase gene, apha-7, was also identified on a 14-kb *C. jejuni* plasmid, pS1178. Kanamycin resistance is often mediated by a plasmid that also encodes tetracycline resistance and has been reported to be transferred along with tetracycline resistance, by conjugation, from representative *C. jejuni* strains to a recipient strain of *C. jejuni* (Gibreel *et al*., 2004). In fact, the race to develop agents to overcome the resistance mechanism is one that man may never win, but the resistance trends should be kept under check through intensive research leading to novel and alternative drugs therapies.

The problems associated with microbial resistance in diarrhoeal patients will continue to pose a challenge to public health workers (Hoge *et al*., 1998). This challenge can be minimized if governments and associated public health services improve water quality and sanitation. This will diminish the transmission of these bacterial pathogens. Misuse of antibiotics has resulted in increased resistance to most of the commonly used drugs for treatment. A call to regulate the use of antimicrobials may be necessary. Development of new vaccines to help reduce the incidence of diarrhoeal disease may be encourageed (Tjaniadi *et al*., 2003).

The World Health Organizations recommends the use of antibiotics only for treatment of the severe diarrhoeal episodes for indigenous children in developing countries. Among travelers the benefits of antibiotic therapy have been well established for non-bloody diarrhoea of a variety of etiologies. A priority in new antibiotic development is to identify agents active against *Campylobacter* species concurrent with the search for effective enteric vaccines.

CONCLUSIONS

There is wide agreement that spread of antibiotic resistance and multiresistance needs to be confronted more than the development of new drugs. There is a need to develop effective strategies to conserve present antimicrobials, to improve infection control and to achieve surveillance of resistance. Resistance and multiresistance to antimicrobial drugs are becoming more prevalent for many common pathogens, and pose serious problems in the hospitals and the community. Limiting the proliferation of resistance will require improved professional and public awareness of appropriate antibiotic usage through

education. In the wake of impending development of resistance in the organisms, search for alternate new drugs should be continued. Accompanied by this strategy, intensive water and sanitation programs and vaccine development would seem to be critical.

Statement of conflict of interest

The authors have declared no conflict of interest.

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