Journal of Health & Population in Developing Countries

Etiology of Salmonellosis in Northern Areas of Pakistan*

Khalil Ahmed, Farah R. Shakooriand A. R. Shakoori**

ABSTRACT

People of northern Pakistan face health hazards because of poor sanitation practices. Bacterial gastrointestinal infections are very common and sometime outbreaks occur. The present study was aimed at evaluating and analyzing infestation of *Salmonella typhi* in stools of patients with suspected gastroenteritis and to ascertain the status of antibiotic therapy. Five hundred and eighty five fecal specimens of suspected gastroenteritis, referred by physicians of District Headquarter Hospital Gilgit, were investigated for common enteropathogenic bacteria from July 1997 to September 1999. Twenty one fecal specimens (3.6%) were found to be infected with *S. typhi*. All the *S. typhi* strains were sensitive to cefotaxime, ceftriaxone, ciprofloxacin and enoxacin. Thirty eight per cent strains were resistant to two antibiotics (ampicillin and chloramphenicol), 24% strains were resistant to ampicillin only and 38% were sensitive to all used antibiotics. A single 23 Kb plasmid was observed in all the ampicillin and chloramphenicol resistant strains. *Escherichia coli* C600 were transformed with the isolated plasmids. All the transformants resisted growth in media containing 10 µg /ml ampicillin and 30 µg /ml chloramphenicol, showing that the antibiotic resistance is mediated on plasmid. It is concluded that in northern Pakistan, the *S. typhi* have developed resistance to ampicillin and chloramphenicol and that the antibiotic resistance is plasmid mediated.

KEY WORDS: Salmonellosis, antibiotic resistance, plasmid borne resistance.

Address for correspondence: Prof. Dr. A. R. Shakoori, Meritorious Professor & Director
School of Biological Sciences, University of the Punjab, New Campus, Lahore 54590, Pakistan.
Phone: 92-42-9230966 Fax: 92-42-9231248; 92-42-9230980
Email: arshak@brain.net.pk

* Part of Ph.D. thesis of first author

** Department of Zoology, Government College, Lahore

The ***Journal of Health and Population in Developing Countries (ISSN 1095-8940)*** is a publication of the Department of Health Policy and Administration, School of Public Health University of North Carolina at Chapel Hill.

UNC SCHOOL OF PUBLIC HEALTH
DEPARTMENT OF HEALTH POLICY AND ADMINISTRATION

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INTRODUCTION

Salmonellosis is an important global public and animal health disease, causing worldwide morbidity and mortality of humans and animals. In the developing countries it is estimated that about 16 million cases of typhoid fever occur annually in the world, causing about 600,000 deaths (Ivanoff, 1995; Parkhill *et al.*, 2001). In the developed countries, on the other hand, it has become a rare disease where transmission is controlled by good hygiene practices and sanitation.

In several parts of the World, where typhoid fever is endemic, there is serious concern about the emerging patterns of S*almonella s*pecies that are resistant to current antibiotics (Khan *et al*., 1996). An increase in the frequency of antibiotic resistance of *Salmonella* isolates have been noted in developed, as well as in developing countries (Cherubin, 1981). The strains of *S. typhi* isolated in the past were sensitive to conventional anti-typhoid drugs, chloramphenicol, co-trimoxazole and ampicillin (Rowe *et al.,* 1987). Over the years, resistant strains of *Salmonella* to all the conventional drugs have emerged. Multi-drug-resistant strains have been reported to be from 20% - 78% in various studies from all parts of the world where typhoid is endemic (Bhutta *et al.,* 1991; Rao *et al.,* 1993). *S. typhi* strains resistant to chloramphenicol, ampicillin and co-trimoxazole have been reported from Calcutta (Anand et *al.,* 1990), Delhi (Gupta e*t al.,* 1990) and Vellore (Jesudason and John, 1990). Shanahan *et al*. (1998) from India isolated 52.38% *S. typhi* strains resistant to chloramphenicol, trimethoprim and amoxicillin and 19% strains resistant to chloramphenicol and trimethoprim. In Bangladesh, the chloramphenicol resistant *S. typhi* was isolated in 1980 from an admitted patient's stool and blood culture (Huq and Samadi, 1982). Albert *et al*. (1990) isolated 16 (12%) multi-drug-resistant *S. typhi* out of 135 *S. typhi;* 1% *S. typhi* was found resistant to ampicillin, co-trimoxazole and chloramphenicol. This resistance increased to 38% in 1993 (Rahman *et al.,* 1994). In Pakistan also the incidence of multi-resistant *S. typhi* has tremendously increased since its first detection in 1987 and now resistance prevalence has increased to more than 80% and even in certain situations to almost 100% (Butt *et al.,* 2000). That the antibiotic resistance is plasmid mediated has been reported in *Salmonella* from different regions of the world (Albert *et al.,* 1990; Rowe *et al.,* 1992; Shanahan *et al.,* 1998).

In Pakistan Salmonellosis is endemic and many studies have been conducted in different parts of the country *e.g.* Karachi (Hafiz *et al.,* 1993; Saqib and Altaf, 2000), Lahore (Khalil *et al.,* 1993), Peshawar (Gandapur *et al.,* 1993) and Rawalpindi/Islamabad (Karamat *et al.,* 1993). No such study has ever been undertaken in the Northern Areas of Pakistan. The people in Northern Areas (1500-8000 m above the sea level) face health hazards because of poor sanitation practices *i.e*. habit of open defecation, lack of hygiene education and use of highly contaminated water. Parasitic and bacterial gastrointestinal infections are very common and some times out breaks occur (Waqar *et al.,* 1999; Ahmed and Shakoori, 2002). This is the first study on the surveillance of salmonellae in the suspected gastroenteritis patients, their antibiotic sensitivity pattern and the transformation of susceptible strain with the antibiotic resistance conferring plasmids.

MATERIALS AND METHODS

Collection of specimens and laboratory examination

Fecal specimens of 585 suspected patients referred to the Laboratory of District Headquarter Hospital Gilgit were collected in clean and open mouthed disposable containers and investigated for salmonellae. The collected fecal specimens were cultured within two hours of their collection. Fecal specimens were analyzed according to WHO Manual for Laboratory Investigations of Acute Enteric Infections (WHO, 1983). Macroscopically fecal specimens were examined for consistency, mucus, blood, parasites and microscopically for pus cells, red blood cells, ova and cysts.

Bacteriological investigation

Fecal specimens were primarily cultured on selective Salmonella-Shigella agar (SSA) and differential medium MacConkey agar. The culture plates were incubated aerobically at 37 ^{0}C for 18 – 24 hours. The fecal specimens were also enriched with Tetrathionate broth (TTB) USA for *Salmonella* enrichment and sub cultured on SSA.

Biochemical identification of Salmonella.

The suspected colonies for *Salmonella* (on MacConkey agar 1-2 mm non lactose fermenting colonies, on SS agar 1-2 mm colorless translucent colonies) from primary culture were biotyped by culturing on Urea solution (SR 20), mixed urea agar base slant, Kligler's iron agar (KIA), Solphide-indole-Motility agar (SIM), and Siminous citrate test agar.

Antibiotic sensitivity test

Antibiotic sensitivity of the identified *Salmonella* was tested according to Bauer *et al*. (Bauer *et al.*, 1966). Commercially available discs (Difco/Oxoid) were used containing ampicillin (10 μg), chloramphenicol (30 μg), ceftriaxone (35 μg), cefotaxime (30 μg), ciprofloxacine (5 μg) and enoxacine (10 μg). Discs were placed on sensitest agar plates which had been swabbed with fresh culture of standard turbidity in order to give a confluent lawn of growth. In all sets of tests, standard *E. coli* ATCC 25922 was used as a control. Sensitivity was scored on the basis of the diameter of the zone of inhibition after overnight incubation at 37 ^{0}C. The antibiotic resistant strains and *Escherichia coli* C600 were further tested by mixing the antibiotics ampicillin (10 μg/ml) and chloramphenicol (30 μg/ml) in the agar medium.

Plasmid analysis and transformation

Plasmid DNA of the antibiotic resistant *S. typhi* strains was isolated according to Holmes (1984) and separated by electrophoresis. The molecular weight of plasmid DNA was estimated by comparing with λ DNA cut with Hind III. The competent cells of the *E. coli* C600 (Cohen *et al.*, 1972), which are plasmid free and sensitive to all the antibiotics, were transformed with the isolated plasmids (Sambrook *et al.*, 1989). The success of transformation was checked by culturing the transformants on antibiotic mixed ampicillin (10 μg/ml) and chloramphenicol (30 μg/ml agar).

Figure 1 Map of Northern Pakistan showing sites from where fecal samples of patients suspected of gastroenteritis were referred to the Laboratory of District Headquarter Hospital, Gilgit for investigation and were found to be infected with salmonellae.

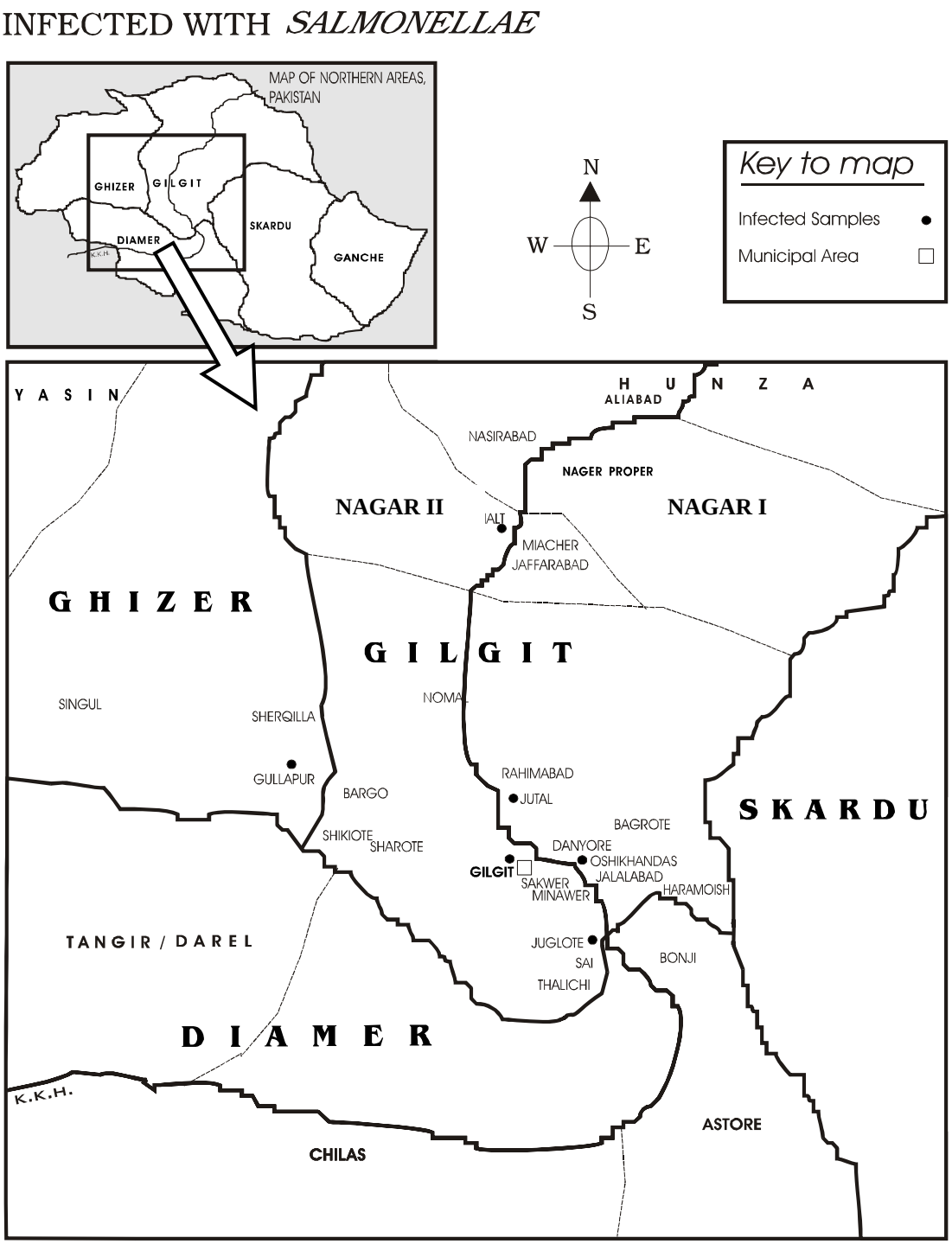


Table 1. Area-wise distribution of suspected gastroenteritis patients referred for bacteriological investigation and infestation of *Salmonella typhi.*

Name of area	Population	Stool samples		Infected samples	
		Total investigated	%	Total infected	%
Gilgit					
Municipal area	56,701	403	0.71	15	3.72
Sakwer	4,553	11	0.24	0	0
Minawer	1,816	2	0.11	0	0
Juglote/Sai	4,201	12	0.29	1	8.4
Nomal	17,774	2	0.01	0	0
Danyore	26,176	83	0.32	0	0
Bagrote	5,962	3	0.05	0	0
Oshikhandas	5,055	4	0.08	1	25
Jalalabad	5,324	1	0.02	0	0
Rahimabad	1,781	5	0.28	0	0
Jutal	2,836	1	0.04	1	100
Bargo	2,964	2	0.07	0	0
Sharote/Shikiote	11,821	12	0.10	0	0
Haramoish	8,024	2	0.03	0	0
Chalt	2,583	6	0.23	1	16.7
Nagar (Jaffarabad)	1,702	1	0.06	0	0
Nagar (Miacher)	2,141	1	0.05	0	0
Nagar proper	29,136	5	0.02	0	0
Nasirabad	3,238	1	0.03	0	0
Sub. Total	**193,728**	**557**	**0.29**	**19**	**3.4**
Ghizer					
Gullapur	10,940	5	0.05	01	20
Sherqilla	11,938	1	0.01	0	0
Singul	10,095	1	0.01	0	0
Yasin	34,391	2	0.01	0	0
Sub. Total	**67,364**	**9**	**0.02**	**1**	**11.1**
Diamer					
Chilas	16,575	6	0.04	0	0
Thilichi	600	1	0.17	0	0
Astore	71,666	2	0.01	0	0
Bonji	11,646	2	0.02	0	0
Sub. Total	**100,487**	**11**	**0.01**	**0**	**0**
Total	**361579**	**577**	**0.16**	**20**	**3.5**
Foreign visitors	-	8	-	01	12.5
G. Total		**585**		**21**	**3.6**

RESULTS

Area-wise distribution and infestation

A total of 577 referred cases of suspected gastroenteritis patients for the bacteriological investigation from different districts of Northern Areas of Pakistan (Fig. 1) were investigated for the infestation of *S. typhi* and 20 (3.5%) were found infected with *S. typhi*. The highest number of specimens were investigated from district Gilgit 557 (0.29%) followed by Ghizer 9 (0.02%) and Diamer 11 (0.01%). The

highest infestation of *S. typhi* was found in Ghizer (11.11%) and Gilgit (3.4%) and no infestation was found in Diamer district (Table1). Eight specimens were also referred from foreign visitors and only one was found infected with *S. typhi* (12.5%).

Table 1 also shows the number of specimens investigated from different localities in each district. In Gilgit specimens were received from 19 localities. Four hundred three specimens were received from Municipal area, which makes it 0.71% of the total population in Gilgit. This was followed by 0.32% from Danyore, 0.29% from Juglote/Sai, 0.28% from Rahimabad, 0.24% from Sakwer, 0.23% from Chalt, 0.11% from Minawer, 0.10% from Sharote/Shikiote, 0.08% from Oshikhandas, 0.07% from Bargo, 0.06% from Jaffarabad, 0.05% each from Bagrote and Miacher, 0.04% from Jutal, 0.03% each from Haramoish and Nasirabad, 0.02% each from Jalalabad and Nagar proper, and 0.01% from Nomal. The infestation of *S. typhi* was found in 5 localities, Jutal (100%), Oshikhandas (25%), Chalt (16.7%), Juglote/Sai (8.4%) and Municipal area (3.72%).

In Ghizer, specimens were received from four localities - 5 cases from Gullapur (0.05%) followed by 0.01% each from Sherqilla, Singul and Yasin. *S. typhi* was found only in Gullapur specimens (20%).

In district Diamer, specimens were received from proper Chilas (0.04%), Thilichi (0.17%), Bonji (0.02%), and Astore (0.01%). No specimen was found infested with *S. typhi.*

Eight cases of foreign visitors were also referred for bacteriological investigation and 1 (12.5%) was found infected.

Year wise and seasonal distribution and infestation

During the entire study from July 1997 to September 1999, out of 585 specimens of suspected gastroenteritis patients 241 were investigated in 1997 (41.20%), 146 in 1998 (24.96%) and 198 in 1999 (33.85%). The *S. typhi* infestation was 1.66% in 1997, 6.85% in 1998 and 3.54% in 1999.

Table 2. Seasonal distribution of suspected gastroenteritis patients referred for bacteriological investigation and infestation of *Salmonella typhi.*

Year	Spring		Summer		Autumn		Winter		Total examined	Total infected (% infestation)
	Samples examined	No. infected (% infestation)	Samples examined	No. infected (% infestation	Samples examined	No. infected (% infestation	Samples examined	No. infected (% infestation		
1977	-	-	90	2 (2.22)	136	2 (1.47)	15	0 (0)	241	4 (1.66)
1988	15	2 (13.33)	81	6 (7.41)	43	2 (4.65)	7	0 (0)	146	10 (6.85)
1999	25	0 (0)	164	7 (4.27)	5	0 (0)	4	0 (0)	198	7 (3.54)
Total	**40**	**2 (5)**	**335**	**15 (4.5)**	**184**	**4 (2.17)**	**26**	**0 (0)**	**585**	**21 (3.6)**

Table 2 shows seasonal distribution and infestation of suspected gastroenteritis patients referred to laboratory for investigation of *S. typhi* and their infestation in each season. From July 1997 to September 1999, 585 cases of suspected gastroenteritis patients were bacteriologically investigated, 335 cases in summer (57.27%), 184 in autumn (31.45%), 40 in spring (6.84%) and 26 in winter (4.45%). The highest infestation of *Salmonella typhi* was found in spring (5%) followed by summer (4.5%) and autumn (2.17%). No *S. typhi* infestation was recorded in winter.

Table 3. Age-wise distribution of suspected gastroenteritis patients referred for bacteriological investigation and infestation of *Salmonella typhi.*

Age group in years	No. of specimens Investigated	No. of specimens infected with *Salmonella typhi* (% infestation)
>0 - 10	321	4 (1.3)
>10 -20	91	3 (3.3)
>20 -30	79	4 (5.1)
>30 -40	40	3 (7.5)
>40 -50	21	3 (14.3)
>50 -60	23	4 (17.4)
>60	10	0 (0.0)
Total	**585**	**21 (3.6)**

Age and sex wise distribution and infestation

Table 3 shows age wise distribution of suspected gastroenteritis patients. The highest number of cases were investigated in age group >0 -10 years (321), followed by >10 - 20 years (91), >20 - 30 years (79), >30 - 40 years (40), >50 - 60 years (23), >40 - 50 years (21) and >60 (10). The highest infestation of *S. typhi* was found in age group >50 - 60 years (17.4%), followed by >40 – 50 years (14.3%), >30 – 40 years (7.5%), >20 – 30 years (5.1%), >10 – 20 years (3.3%) and >0 – 10 years (1.3%).

Out of 585 investigated cases 391 (67.35%) were male and 191 (32.62%) were female, the infestation of *S. typhi* in them was respectively 3.3% and 4.2%.

Antibiotic resistance pattern

Table 4 shows multiple antibiotic resistance of *S. typhi* against different antibiotics. 38 % strains were found resistant to double antibiotics (ampicillin and chloramphenicol) and 24% strains with only ampicillin and 38% strains were sensitive to all the tested antibiotics. No strain was resistant to cefotaxim, ceftriaxone, ciprofloxacin and enoxacin.

Table 4. Multiple antibiotic resistance of *Salmonella typhi* isolated from fecal samples of suspected gastroenteritis patients in the Northern Areas of Pakistan.

Resistant to	Resistance pattern	No. of resistant strains	Percentage
Single drug	Ampicillin	5	23.81
Two drugs	Ampicillin, chloramphenicol	8	38.1
Sensitive to all used antibiotics	Ampicillin, chloramphenicol, Ceftriaxone, cefotaxim and Enoxacin	08	38.1

Antibiotic resistance gene

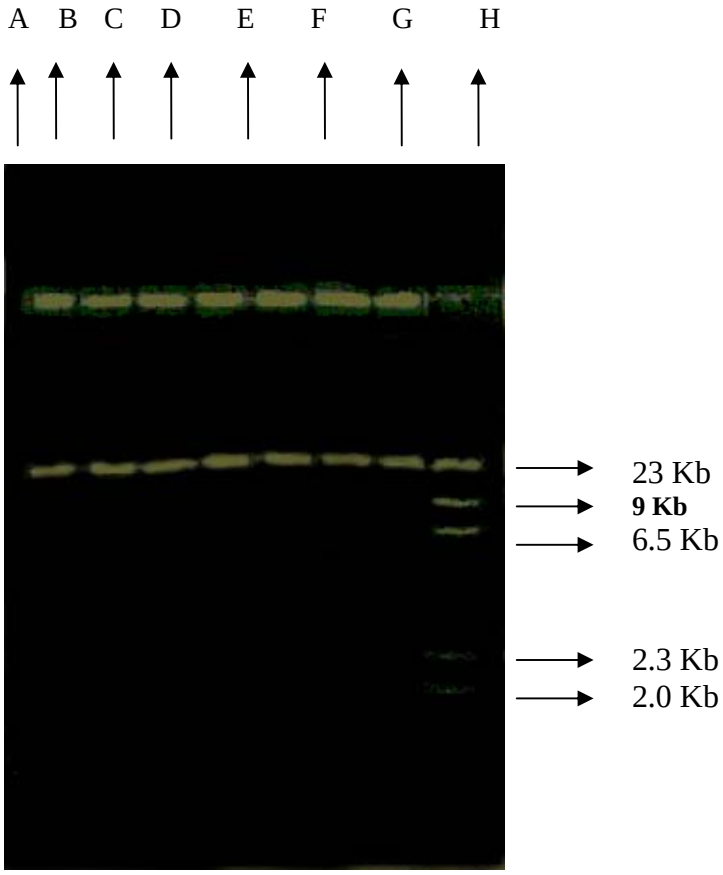
Thirteen antibiotic resistant *S. typhi* strains were processed for plasmid isolation and a 23 Kb plasmid was found in all the strains (Fig. 2).

Table 5 shows transformation of antibiotic resistant plasmid of *S. typhi* into *Escherichia coli* C600 (sensitive to all antibiotics). Plasmids of 13 antibiotic resistant strains (8 with both ampicillin and chloramphenicol and 5 only with ampicillin) were processed for transformation. All the transformants resisted growth on L. B. agar plates containing ampicillin (10 µg/ml) and chloramphenicol (30 µg/ml).

Table 5. Transformation of antibiotic resistance gene in *Salmonella typhi*.

Name of antibiotics	No of strains processed for plasmid isolation	Transformed	
		Amp, C	Amp
Ampicillin (Amp).	5	-	5 (100%)
Ampicillin, chloramphenicol (Amp,C).	8	8 (100%)	-
Total	**13**	**8 (100%)**	**5 (100%)**

Figure 2. Plasmid profile of *Salmonella typhi* isolated from fecal samples of suspected gastroenteritis patients in the Northern Pakistan. A 23 Kb plasmid is visible in Lane A (*S. typhi* 20), B (*S. typhi* 134), C (*S. typhi* 149), D *(S. typhi* 209), E (*S. typhi* 263), F (*S. typhi* 310) and G (*S. typhi 312)*. Lane H shows marker λ DNA cut with Hind III.



DISCUSSION

Salmonella typhi remains an important enteric pathogen with an annual global burden of approximately 16 million cases, and 600,000 fatalities (Parkhill *et al.*, 2001). Outbreaks of typhoid fever have been caused by antibiotic resistant *S. typhi*, such as in Mexico in the early 1970s (Datta and Olarte, 1974).

Typhoid fever is a public health problem. It is endemic in Pakistan and will remain as long as lack of economic development allows the organism to flourish. According to our study the infestation of *S. typhi* is 3.59%, which is exactly equal to that of Saqib and Altaf (2000), observed in their study in Karachi, but comparatively lower than the other studies conducted in the country and higher than the developed countries. In Karachi, Hafiz *et al*. (1993), isolated 6.7% salmonellae from 25,000 blood cultures and 92% of the total isolates were *S. typhi*. Karamat *et al.* (1993), on the other hand, isolated 22.13% different types of salmonellae from Rawalpindi / Islamabad areas and Yamashiro *et al*. (1998), in their study in Vientiane, Lao people's Democratic Republic isolated only 0.6% *S. typhi*.

Our study data represents the referred cases and area-wise data indicate that more specimens were referred from areas close to the hospitals and therefore, higher infestation of *S. typhi* was found in areas closer to the Hospital. This high number of cases from the nearby areas may be because of two reasons: (1) The physicians refer more patients of nearby areas to avail the laboratory facilities and for the far-flung areas the patients do not wait for the laboratory findings therefore physicians treat them with out laboratory investigations, (2) In the peripheries the physicians treat the normal gastroenteritis patients and refer only those patients who are not cured by them.

Year-wise investigated data indicated that low number of cases was referred and high infestation of *S. typhi* was found in the year in which no outbreak of *V. cholerae* occurred. This is because during outbreak years, the cases more strongly suspected for *V. cholerae* were referred to the laboratory.

In our study highest infestation of *S. typhi* was observed during the spring and summer seasons. In India, Sinha *et al*. (1999) also found highest incidence of typhoid in the monsoon season. The typhoid is water- and food-borne disease, and an average temperature of 35 ^{0}C during summer provides optimum conditions for the growth of bacteria.

S. typhi infestation has been recorded in all age groups which tend to increase with increasing age. Our findings contradict the findings that typhoid is a common and significant cause of morbidity between 1 and 5 years of age (Sinha *et al.*, 1999), and also the view that typhoid peaks between 5 and 12 years (Patniak and Kapoor, 1967). Our findings support the current view that typhoid in children under 5 years is mild.

Although the isolated salmonellae were previously found to be uniformly sensitive to conventional drugs *viz.*, chloramphenicol, co-trimoxazole and ampicillin (Rahman *et al.*, 1994), the resistant strains of salmonellae to commonly used drugs have gradually emerged over the years. Multi drug resistant strains have been reported, 20% - 78% in various studies, from all parts of the world where typhoid is endemic, (Albert *et al.*, 1990; Butt *et al.*, 2000). In Ontario, Canada, Harnett *et al*. (1998), studied the sensitivity of 214 strains of *S. typhi* to 20 antimicrobial agents. Among them 48 of the 214 isolates 22.4% were from individuals who had traveled in South Asia, were multi resistant and more than 91% were resistant to ampicillin, chloramphenicol, tetracycline, streptomycin, sulfamethoxazole, trimethoprim, co-trimoxazole and piperacillin. In Pakistan also multi-resistant *Salmonella typhi* have been isolated during the past few years (Hannan *et al.*, 1991). Bhutta (1996) found 32% multi-drug-resistant salmonellae strains from Karachi, and Mirza *et al*. (1993) isolated 25 *S. typhi* from typhoid fever patients from 1990 to 1991 in Rawalpindi and Islamabad. All isolates were resistant to chloramphenicol and ampicillin. In our study 38% of the isolated *S. typhi* were resistant both to ampicillin and chloramphenicol and 23% to ampicillin only. Majority of the findings in this study were similar to those reported from other laboratories (Raymond *et al.*, 1987; Bhutta *et al.*, 1991), most of the isolates having developed resistance against the first line of drugs. It is thought that increasing resistance to ampicillin and chloramphenicol is due to unrestricted antimicrobial use in our country [Raymond *et al.*, 1987). Typhoid fever poses a threat and is a cause of high morbidity and mortality in the tropical countries. Over the last decade there has been a steady increase in the development of multiresistant strains of salmonellae all over the world. In a recent study in Pakistan multiresistant strains of salmonellae has increased to over 39% (Hafiz *et al.*, 1998).

In India, in an epidemic study Karmaker *et al*.(1991) isolated *S. typhi* of which 84% were resistant to ampicillin, chloramphenicol and all were sensitive to ciprofloxacin. In multiple antibiotic resistance strains they found 120 kb and 14 Kb plasmids, and no plasmid was observed in the sensitive strains and

the antibiotic resistance was mediated on 120 Kb plasmid. In UK, Rowe *et al*. (1992) reported the plasmid mediated resistance of chloramphenicol and ampicillin in *S. typhi*. In India, Shanahan *et al*. (1998) found that out of 21 *S. typhi* strains, 15 (71.43%) were resistant to different antibiotics and that the resistance was plasmid mediated. In Bangladesh, Rahman *et al*. 1994) studied from 1989 to 1993 the increase of antibiotic resistance in *S. typhi* against ampicillin, co-trimoxazole, chloramphenicol and found that this resistance was mediated on 110 Mda transferring plasmid.

In this study all the antibiotic resistant *S. typhi* strains were found to harbor single 23 Kb plasmid which mediated antibiotic resistance. Mirza *et al*. (1993) in their study also isolated single 98 MDa self-transferable plasmid with antibiotic resistance for ampicillin, chloramphenicol, tetracycline, streptomycine, sulphamethoxazole and trimethoprim.

It is concluded that *S. typhi* infestation was high (6.85%) in 1988 as against 1.66% in 1997 and 3.54% in 1999. The highest infestation was found in spring (5%) followed by summer (4.5%). Thirty eight per cent strains were found to be resistant to ampicillin and chloramphenicol, and 24% to ampicillin alone. The antibiotic resistance was found to be plasmid mediated.

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