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Emergence of Multidrug-resistant *Salmonella* Gloucester and *Salmonella* Typhimurium in Bangladesh

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ABSTRACT

Infections due to non-typhoid *Salmonella*, resistant to antibiotics, have recently emerged as an important health problem worldwide. Antibiotic resistance was studied by the disc-diffusion method among 3,876 (2.78%) non-typhoid *Salmonella* isolates cultured from 139,279 faecal samples in a diarrhoea treatment centre in Dhaka, Bangladesh, during 1989-1996. Of 499 salmonellae isolated in 1989, serogroup C (1.12%) was the most common, followed by *Salmonella* Typhi (0.72 %) and serogroup B (0.71%). Isolation rate of serogroup B increased significantly to 2.18% ($p<0.01$) in 1992 compared to 0.56% in 1991, 2.86% in 1995, and 2.48% in 1996. Serotyping of 194 serogroup B isolates revealed *Salmonella* Typhimurium (52%) and *Salmonella* Gloucester (45%) as predominant serotypes. Resistance to ampicillin (A), chloramphenicol (C), and trimethoprim-sulphamethoxazole (Sxt) (R type-ACSxt) increased to 89-100% during 1992-1996 from 20-28% during 1989-1991 ($p<0.01$) among *S.* Typhimurium and *S.* Gloucester isolates. In 1993, 8-10% of the strains of both the serotypes, resistant to ampicillin, chloramphenicol, and trimethoprim-sulphamethoxazole, acquired resistance to ceftriaxone (Cr) (R type-ACSxtCr), which increased to 85-92% in 1996 ($p<0.01$). All were susceptible to ciprofloxacin. A 157-kb conjugative plasmid transferred R type-ACSxt from both the serotypes to *Escherichia coli* K-12. The findings of the study suggest the emergence of multidrug-resistant *S.* Gloucester and *S.* Typhimurium for the first time as a significant health problem in Bangladesh, and surveillance is essential to monitor the resistant non-typhoid *Salmonella* and identify its sources and modes of transmission.

Key words: *Salmonella*; *Salmonella* infections; *Salmonella* Gloucester; *Salmonella* Typhimurium; Drug resistance, Microbial; Antibiotic resistance; Plasmid; Bangladesh

INTRODUCTION

Non-typhoid *Salmonella* infections continue to be an important health problem worldwide, resulting in considerable morbidity and occasionally death, especially in extremes of age, immuno-compromised patients, and recently in otherwise healthy patients (1-4). Antimicrobial therapy is indicated for invasive non-typhoid *Salmonella* infections.

In the past few years, there has been a significant increase in multidrug-resistant non-typhoid *Salmonella* infections, including invasive infections throughout the world with significant impact on public health and healthcare cost in many countries (4-7). Epidemic spread of multidrug-resistant *Salmonella* Typhimurium continued to occur in India since 1978 (4). Recently, *S.* Typhimurium DT104, resistant to ampicillin, chloramphenicol, tetracycline, sulphonamide, and streptomycin, was reported to cause epidemics of

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salmonellosis in humans and animals in the UK, France, and other European countries (2,5). Infections, caused by five drug-resistant *S*. Typhimurium DT104, were associated with greater morbidity and mortality than other *Salmonella* infections in the UK: 41% of DT104 cases were hospitalized, and 10 (3%) of 295 culture-confirmed cases died (5). Glynn *et al*. reported a significant increase in the prevalence of multidrug-resistant *S*. Typhimurium DT104 in the USA (~300,000 cases per year) and described it as a rapidly-emerging pathogen (6).

There is evidence now that this epidemic strain has spread efficiently through the food chain in the USA (8), Canada (9), and Europe (10) posing a significant threat to public health. Of further concern is that the strains, isolated in the UK, acquired additional resistance to trimethoprim and ciprofloxacin (11).

Non-typhoid *Salmonella* infections are common in Bangladesh (12,13). *S.* Virchow, *S.* Veltervreden, *S.* Typhimuriun, and *S.* Gloucester were identified as frequently-isolated serotypes during 1988-1989 by analyzing 14 representative non-typhoid *Salmonella* in Clinical Research and Service Centre (CRSC) of ICDDR,B: Centre for Health and Population Research (13). In 1994, Hoque *et al*. reported 3 cases of fatal extra-intestinal infections, such as meningitis, bronchopneumonia, septicaemia, and urinary tract infection, in children caused by multidrug-resistant *S.* Gloucester (7). Recent information on the prevalence of different serogroups and predominant serotypes of non-typhoid *Salmonella* is not available in Bangladesh. Besides, the emerging problem of antimicrobial resistance among recent non-typhoid *Salmonella* isolates in Bangladesh is not well-documented.

We report here a sustained increase in the isolation rate of multidrug-resistant non-typhoid *Salmonella* spp. that pose a serious threat to public health.

MATERIALS AND METHODS

Clinical samples

The study was conducted in Clinical Microbiology Laboratory of the CRSC, ICDDR,B. The Centre serves over 100,000 diarrhoeal patients annually. All *Salmonella* isolates, obtained by culturing faecal samples in the Clinical Microbiology Laboratory from 1989 to 1996, were included in the study. The sources of faecal samples were: a 2%-subsample of diarrhoeal patients (every 50th patient) attending the CRSC, Dhaka, and patients whose clinical conditions require stool cultures as suggested by physicians and submitted to the above-mentioned microbiology laboratory.

Microbiological techniques

The faecal samples were cultured onto MacConkey and Salmonella-Shigella agar, and in sellinite F enrichment broth for isolation of salmonellae (13). Suspected bacterial colonies were identified by standard biochemical tests, and were then serogrouped and serotyped by slide agglutination using *Salmonella* O and H group antisera (Difco Laboratories, Detroit, MI, USA) by standard method (7). Selected isolates of serogroup B (on an average 24 per year for 8 years) were further serotyped. Antimicrobial susceptibility was determined by the disc-diffusion technique (14), using Mueller-Hinton agar, commercial antibiotic discs (Oxoid, Basingstoke, UK) and *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923) as control strains. The following commercial discs were used: ampicillin (A, 10 µg), chloramphenicol (C, 30 µg), trimethoprim-sulphamethoxazole (Sxt, 1.25 µg/ 23.75 µg), ceftriaxone (Cr, 30 µg), and ciprofloxacin (Cip, 5 µg).

Plasmid DNA was extracted from multidrug-resistant *S*. Typhimurium (n=12, R-type ACSxtCr) and *S*. Gloucester (n=12, R-type ACSxtCr) serotypes according to the method of Portnoy *et al*. (15). Briefly, an isolated colony of each strain was grown in 3 mL Bain Heart infusion broth (BHI, Gibco BRL, USA) overnight in air at 37 ºC. The cells were collected, lysed by lysis buffer (50 mM Tris-HCl, 10 mM EDTA, 4% SDS, P^H 12.4). DNA from the supernatant was precipitated with an equal volume of cold (-20 ºC) isopropanol. Plasmid DNA was separated by electrophoresis in 0.7% agarose gel. The gel was run for 3-4 hours at 55 volts (90 amperes) for a gel of 15 samples (14x12 cm). Reference plasmid markers, V517 and 39R861, were used for determining the size of unknown plasmids. Gels were stained with ethidium bromide and visualized by ultraviolate transilluminator for plasmid DNA. The transfer of R plasmid was determined by conjugation between multidrug-resistant *S*. Typhimurium and *S*. Gloucester serotypes and recipient *E. coli* K12 (F-, lac-, Rif^R) according to the method of Neu *et a1*. (16). The recipient *E. coli* K12 was susceptible to all drugs, except rifampicin. Transconjugants were selected on brain-heart infusion agar containing rifampicin (200 µg/mL) and chloramphenicol (125 µg/mL) or tetracycline (100 µg/mL). All putative transconjugants

were tested for antimicrobial susceptibility, plasmid profiles, and lactose-fermenting property to select transconjugants and to differentiate from spontaneous rifampicin-resistant donor mutant. Statistical analysis was done by chi-square test.

RESULTS

The frequency of isolation of salmonellae from faecal samples during 1989-1996 and distribution of common serogroups are summarized in Table 1. In total, 4,818 (3.46%) *Salmonella* strains were isolated from 139,279 faecal samples during 1989-1996, of which 942 (0.68%) were typhoid salmonellae, and the remaining 3,876 (2.78%) were non-typhoid salmonellae. In 1989, *Salmonella* serogroup C (1.12%) was the most common isolate, followed by *S.* Typhi (0.72%) and serogroup B (0.71%). The isolation rate of group B did not increase till 1991. In 1992, the isolation rate of *Salmonella* serogroup B increased to 2.18% ($p<0.01$) compared to 0.56% in 1991 and increased further to a peak of 2.86% in 1995, and decreased to 2.48% in 1996. In contrast, the isolation of typhoid salmonellae and other non-B serogroups of non-typhoid salmonellae remained the same or decreased during the next seven years (1990-1996) compared to those of 1989.

Table 1. *Salmonella* isolates from faecal samples by year, 1989-1996

Year	No. of faecal samples	No. of *Salmonella* isolates	No. of non-typhoid salmonellae (n=3,876)			No. of typhoid salmonellae (n=942)	
			Group B	Group C	Others	*S.* Typhi	*S.* Paratyphi
1989	14,666	499 (3.4)*	104 (0.71)	164 (1.12)	94 (0.64)	106 (0.72)	31 (0.21)
1990	10,163	251 (2.5)	73 (0.72)	63 (0.62)	59 (0.58)	44 (0.43)	12 (0.12)
1991	14,827	350 (2.4)	83 (0.56)	101 (0.68)	62 (0.42)	93 (0.63)	11 (0.07)
1992	16,392	628 (3.8)	357 (2.18)	117 (0.71)	62 (0.38)	84 (0.51)	8 (0.05)
1993	22,668	608 (2.7)	240 (1.06)	113 (0.50)	122 (0.54)	121 (0.53)	12 (0.05)
1994	19,647	731 (3.7)	300 (1.53)	142 (0.72)	112 (0.57)	163 (0.83)	14 (0.07)
1995	22,126	977 (4.4)	633 (2.86)	104 (0.47)	101 (0.46)	127 (0.57)	12 (0.05)
1996	18,790	774 (4.1)	466 (2.48)	104 (0.55)	100 (0.53)	95 (0.50)	9 (0.05)
Total	139,279	4,818 (3.46)	2,256 (1.62)	908 (0.65)	712 (0.51)	833 (0.60)	109 (0.08)

* Figures in parentheses indicate isolation rates

To address the emerging problem of non-typhoid salmonellae, hereafter, we will refer only to non-typhoid salmonellae in this paper. To determine the important serotypes of frequently-encountered serogroup B, further serotyping of 194 (range 22-30 isolates per year for 8 years) serogroup B isolates during 1989-1996 revealed that *S.* Typhimurium (52%) and *S.* Gloucester (45%) serotypes were predominant, comprising 97% of the serogroup B isolates.

Overall, 45% (1,739 of 3,876) of non-typhoid *Salmonella* isolates were resistant to one or more antibiotic(s). Thirty-nine percent (1,510 of 3,876) of all the isolates were resistant to multiple drugs (resistant to 3 or more unrelated antibiotics), and 3.2% and 2.7% were resistant to two and one drug(s) respectively. Forty-four percent of all non-typhoid salmonellae were resistant to ampicillin, 39% to chloramphenicol, 42% to trimethoprim-sulphamethoxazole, 10% to ceftriaxone, and none to ciprofloxacin. Of all the isolates, only serogroup B was significantly more resistant to antibiotics than serogroup C and other serogroups (Table 2). The incidence of antibiotic resistance changed significantly only among serogroup B isolates. In 1989, 15% (16 of 104) of *Salmonella* serogroup B isolates were resistant to one or more antibiotic(s) that increased to 22% (18 of 83) in 1991. However, an abrupt upsurge of resistance to 78% (278 of 357 serogroup B) was observed in 1992 ($p<0.01$) compared to 1991, which further increased to 81% (378 of 466) in 1996 due to increased isolation of multidrug-resistant *S.* Typhimurium and *S.* Gloucester serotypes in 1992 and onward. The incidence of resistance for non-B serogroups (serogroup C and others) did not change significantly compared to serogroup B (Table 2) during the study period. Among the non-typhoid *Salmonella* serogroup B isolates, there has been an alarming increase in the incidence of resistance (range 78-80%) to ampicillin, chloramphenicol, trimethoprim-sulphamethoxazole, and ceftriaxone in 1996 compared to 1989 (range 0-15%, Table 3). In contrast, the incidence of resistance to these

drugs either remained more or less the same or increased to a lesser extent among non-B serogroup salmonellae during the study period.

tested in that year acquired additional resistance to ceftriaxone (R type-ACSxtCr), which increased abruptly to 85% (11 of 13 isolates, p<0.01) in 1996.

Table 2. Frequency of antibiotic resistance* among non-typhoid *Salmonella* isolates (n=3,876), 1989-1996

Serogroup	Percentage of resistant isolates by year by group								Overall
	1989	1990	1991	1992	1993	1994	1995	1996	%
B	15	26	22	78	65	62	83	81	70
C	7	13	7	5	5	9	9	9	8
Others	4	25	13	11	14	18	7	5	12
All	9	21	13	54	38	39	66	58	45

*Resistance to one or more antibiotic(s) teste

Antibiotic resistance was determined among 91 *S.* Gloucester and 101 *S.* Typhimurium obtained during 1989-1996. Eighteen percent (2 of 11 isolates tested) of *S.*Gloucester isolates in 1989 (Fig. 1) were simultaneously resistant to ampicillin, chloramphenicol, and trimethoprim-sulphamethoxazole (R-type ACSxt). In 1992, 89% (8 of 9 isolates tested) of *S.* Gloucester isolates had R-type ACSxt (p<0.01, compared to 1989), and all (13 of 13 isolates tested) had the same R-type in 1993. No ceftriaxone resistance was detected till 1992, and 10% (1 of 10) of *S.* Gloucester isolates tested in 1993 acquired additional resistance to ceftriaxone (R-type ACSxtCr) that increased abruptly to 92% (11 of 12 isolates, p<0.01) in 1996. In 1989, 22% (2 of 9) of *S.* Typhimurium isolates were simultaneously resistant to three drugs (R-type ACSxt) that increased to 95% (p<0.01) in 1992. Ceftriaxone resistance was detected in 1993 among *S.* Typhimurium, and 8% (1 of 13) isolates

The *S.* Typhimurium isolates with R-type ACSxtCr had identical plasmid patterns. All 12 strains tested had 5 plasmids: 157, 72, 6, 3.5 and 1.5 kb (Fig. 2). In contrast, 12 *S.* Gloucester isolates with R-type ACSxtCr had 157, 72, 3.5, and 1.5-kb plasmids. Resistance to ampicillin, chloramphenicol, and trimethoprim-sulphamethoxazole in both *S.* Typhimurium and *S.* Gloucester serotypes were transferred to *E. coli* K12 by conjugation and were mediated by 157-kb conjugative R plasmid.

Table 3. Frequency of resistance to commonly-used antibiotics among non-typhoid *Salmonella* isolates (n=3,876), 1989-1996

Antimicrobial agent*	Serogroup	Percentage of resistant isolates by year							
		1989	1990	1991	1992	1993	1994	1995	1996
Ampicillin	B	15	23	20	77	65	65	84	81
	C	6	13	5	4	4	6	4	7
	Others	4	25	11	8	12	15	11	5
Chloramphenicol	B	7	17	12	69	57	59	82	79
	C	3	6	3	1	0	0	1	2
	Others	2	8	8	6	7	8	1	1
Trimethoprim-sulphamethoxazole	B	11	23	11	71	61	61	84	80
	C	2	9	5	3	3	6	8	1
	Others	2	24	8	10	13	11	9	3
Ceftriaxone	B	0	0	0	0	4	20	80	78
	C	0	0	0	0	0	0	1	0
	Others	0	0	0	0	0	1	0	0

*Ciprofloxacin resistance was not detected in the study by the disc-diffusion technique

DISCUSSION

A few studies have examined the frequency of antibiotic resistance among non-typhoid *Salmonella* isolates from humans in Bangladesh. The present study demonstrates for the first time that infection (gastroenteritis) due to multidrug-resistant non-typhoid *Salmonella* serogroup B is a significant and a rapidly-emerging health problem in Bangladesh since 1992. Although the overall isolation

rates of *Salmonella* from faecal samples did not change significantly during the study period (1989-1996) in our laboratory, the isolation rate of serogroup B had four-fold increase in 1992 compared to 1991 and remained high since then. In contrast, the isolation of non-B serogroups of non-typhoid *Salmonella* either decreased or remained the same, or increased to a lesser extent during the study period.

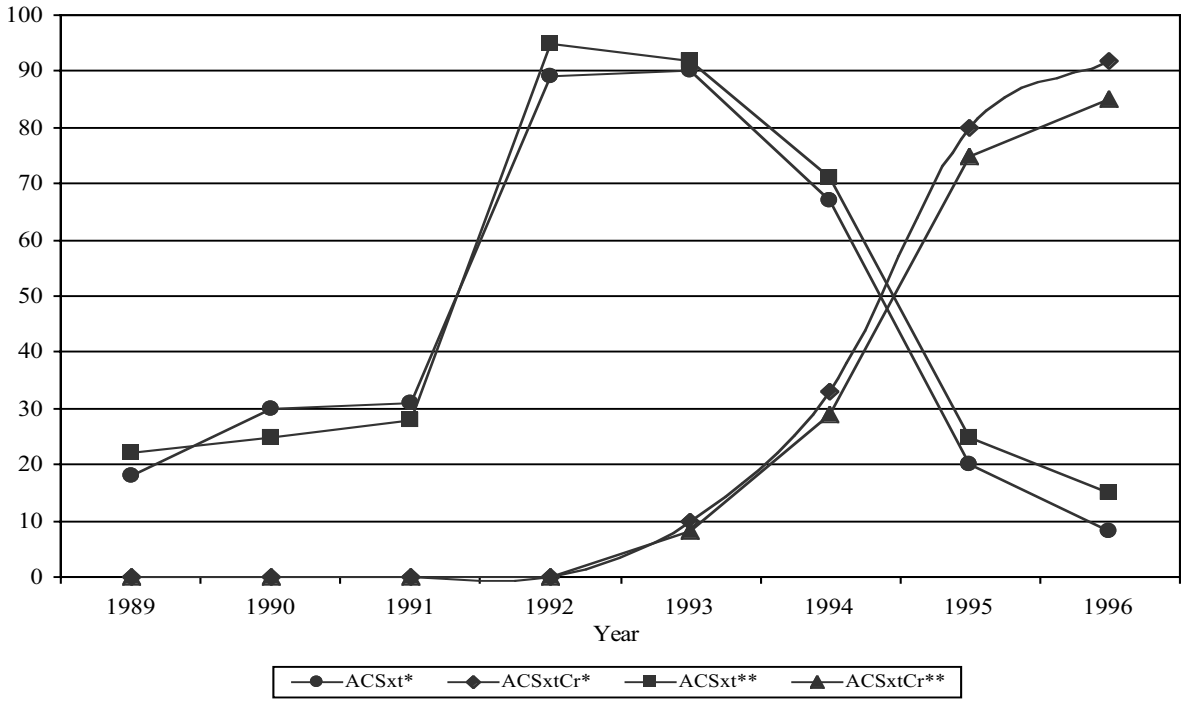

Fig. 1. Frequency of ampicillin (A), chloramphenicol (C), trimethoprim-sulphamethoxazole (Sxt) (ACSxt) and ceftriaxone (ACSxtCr) resistance patterns among *S.* Gloucester* and *S.* Typhimurium**, 1989-1996

Although we could not serotype all serogroups of non-typhoid salmonellae, the results of serotyping of a limited number of *Salmonella* serogroup B isolates and the unique three-drug resistance pattern revealed that *S.* Typhimurium and *S.* Gloucester were actually the underlying cause of the emergence of multidrug-resistant serogroup B *Salmonella* infections in Bangladesh. The proportion of isolates with the three-drug resistance pattern increased from 7% in 1989 to 78% in 1996 among *Salmonella* serogroup B with an additional resistance to ceftriaxone (R-type ACSxtCr). Most of these isolates are likely to be *S.* Typhimurium and *S.* Gloucester. An earlier study also reported *S.* Typhimurium and *S.* Gloucester as frequent isolates from human faecal samples in Bangladesh, by analyzing a small number of isolates but their antimicrobial resistance patterns were not determined (13). Interestingly, the frequency of antibiotic resistance remained relatively low among non-B serogroups of non-typhoid *Salmonella* during the study period (1989-1996). The rapid increase in resistance to ampicillin, chloramphenicol, trimethoprim-sulphamethoxazole, and ceftriaxone is of particular concern, since these drugs are frequently used for treating a wide range of bacterial infections, including *Salmonella* infections.

The study has some limitations. First, the study was based on isolates from the laboratory that might represent a selected sample and could bias the isolation rate of non-typhoid salmonellae and the frequency of antimicrobial resistance. Second, no attempt was made to collect population-based samples from representative areas of the country. Third, we could not serotype all non-typhoid isolates. Finally, clinical features and outcome of multidrug-resistant non-typhoid infections were not studied.

Factors contributing to the overall increase in antibiotic resistance among non-typhoid salmonellae are

many. These include an increase in the isolation rate of multidrug-resistant *S.* Typhimurium and *S.* Gloucester serotypes (serogroup B), the presence of a conjugative R plasmid encoding and disseminating multidrug resistance trait, and selective pressure of antibiotics resulting primarily from inappropriate use of antibiotics. Prior exposure to an antibiotic before the onset of salmonellosis is a risk factor for having a resistant *Salmonella* infection (1). An antibiotic may act specifically and non-specifically to favour intestinal colonization with resistant *Salmonella* by lowering the infective dose for infection or by converting an asymptomatic infection to clinical illness (17).

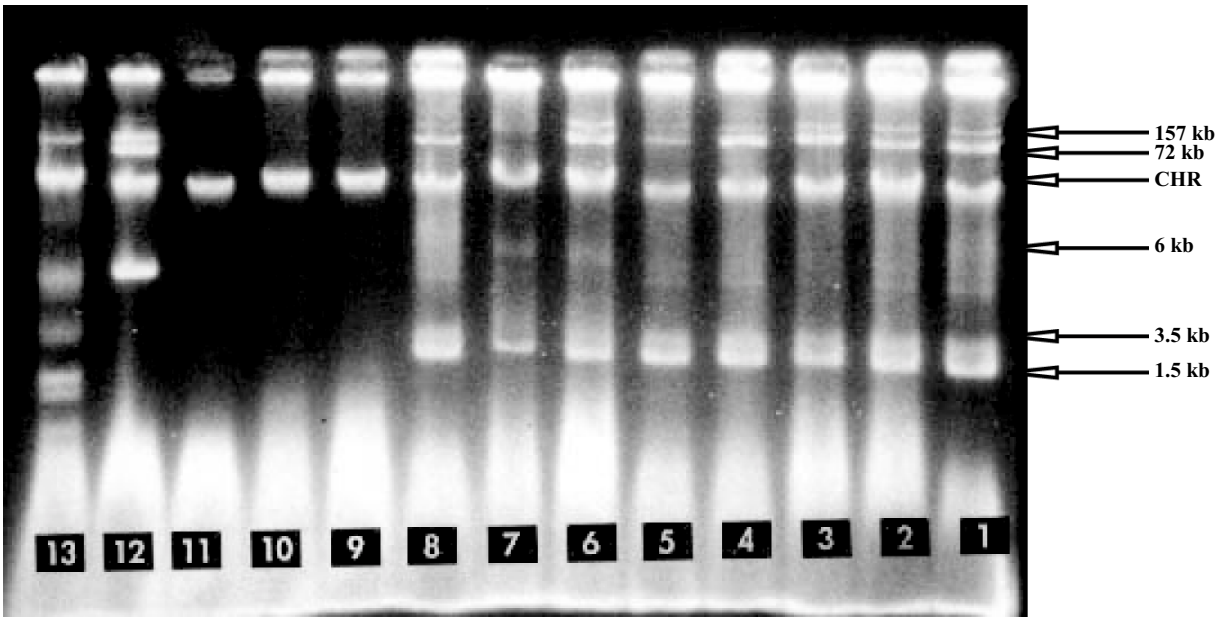

Fig 2. Plasmid profiles of *Salmonella* Typhimurium, *Salmonella* Gloucester, and other *Salmonella* spp. Lane 1,6,7,8: *S.* Typhimurium; Lane 2,3,4,5: *S.* Gloucester; Lane 9,10: Other *Salmonella* spp.; Lane 11: *Escherichia coli* K-12; Lane 12: 39 R861 (Plasmid marker); Lane 13: V517 (Plasmid marker)

The emergence of antibiotic resistance in non-typhoid *Salmonella* isolates in Bangladesh is likely to be associated with therapeutic and non-therapeutic utilization of antibiotics in animals used as human food. Many scientists are concerned that the use of antibiotics to prevent and treat disease in animals and to enhance growth may apply selective pressures, leading to the emergence of antibiotic-resistant organisms that can subsequently cause infections in humans. In 1997, a group of experts from the World Health Organization made recommendations to minimize the further emergence of resistance to antibiotics by promoting the prudent use of antibiotics in food animals. The group recommended ending the use of antibiotics as growth-promoting agents in animals that were used in human medicines. These antibiotics include tetracycline and penicillin, to which salmonellae are resistant. We could not detect any ciprofloxacin-resistant non-typhoid *Salmonella* isolates in the study by the disc-diffusion technique. In the UK in 1996, 14% of five-drug-resistant *S.* Typhimurium DT104 isolates were also resistant to ciprofloxacin (11).

The sources of resistant *S.* Typhimurium and *S.* Gloucester serotypes remain to be determined in Bangladesh. In India, UK, and Canada, the resistant *S.* Typhimurium serotype is widely distributed in food animals, particularly cattle, although isolations from sheep, goats, turkeys, and pigs and from a wide range of food products and processed foods are increasing (2,4,9). Studies in the USA have also shown associations between *S.* Typhimurium DT104 infections in humans and the consumption of non-pasteurized diary products and direct contact with livestock (8). Epidemiological evidence suggests that the main vehicle of transmission of non-typhoid *Salmonella* to humans is food (2). Further studies are urgently required to determine the common source and mode of transmission of multidrug-resistant

S. Typhimurium and *S.* Gloucester in Bangladesh to contain these multidrug-resistant pathogens.

The resistance phenotype ACSxt was encoded by a conjugative 157-kb R plasmid in both the multidrug-resistant *Salmonella* serotypes. A conjugative R plasmid of similar size was identified in gut flora of healthy children in Bangladesh and encoded resistance to ampicillin, chloramphenicol, and trimethoprim-sulphamethoxazole, suggesting the potential role of a promiscuous plasmid in acquisition and dissemination of multidrug resistance traits maintaining an antibiotic resistance gene pool among bacterial population (18).

From 1969 to 1998, considerable clinical and epidemiological evidence indicated that, globally, the emergence and prevalence of some epidemic strains of human *Salmonella* spp. correlated with the acquisition of conjugative or non-conjugative plasmids conferring resistance to multiple drugs and ranging in size from 100 to 180 kb (19,20). Recently, Tosini *et al*. (21) detected a 140-kb conjugative multidrug resistance plasmid in *S.* Typhimurium isolated from patients with gastroenteritis in Albania. In contrast, multidrug resistance traits were mediated by non-autotransferring plasmid in *S.* Typhimurium in India (22) and by chromosome in *S.* Typhimurium DT104 isolates in the UK (10). However, antibiotic resistance genes in multidrug-resistant *S.* Typhimurium DT104 isolates from Europe, USA, and other countries and in non-DT104 isolates from Europe are located in integrons and transposons (21,23)). We do not know whether integrons hosting multidrug-resistant genes are also present on 157-kb conjugative R plasmid in multidrug-resistant *S.* Typhimurium and *S.* Gloucester in Bangladesh.

We have not collected data on clinical illness in the present study on antibiotic resistance, and there remains much to be learnt about the spectrum of illness caused by multidrug-resistant *S.* Gloucester and *S.* Typhimurium. However, most *Salmonella*-positive cases presented with gastroenteritis in our treatment centre. Hoque *et al*. reported severe extra-intestinal infections caused by *S.* Gloucester in Bangladesh that were associated with high case-fatality (7). Fluoroquinolone, such as ciprofloxacin, may be considered for invasive multidrug-resistant *Salmonella* infections in Bangladesh, although reduced susceptibility to it has been reported elsewhere (24). The development of fluoroquinolone resistance among these multidrug-resistant *Salmonella* serotypes that cause invasive human illness would have serious public-health implications.

The study has shown that infections due to multidrug-resistant *S.* Gloucester and *S.* Typhimurium are an important health problem in Bangladesh. Although the infection is common, it is difficult to measure the real disease burden caused by these two serotypes, because almost no laboratory routinely cultures and serotypes *Salmonella* isolates in Bangladesh. Continued and specific surveillance is necessary to monitor and investigate this problem. The sources of infection, mode of transmission, and risk factors for infection with multidrug-resistant *Salmonella,* particularly newly-emerged *S.* Gloucester, must be identified to contain this infection before its widespread transmission to other countries. Appropriate use of antibiotics in humans and farm animals needs to be addressed in Bangladesh and in other countries.

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