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Prevalence and Antimicrobial Resistance of *Salmonella* strains Isolated from Blood Cultures in Dhaka Division, Bangladesh

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A total of 1050 *Salmonella* isolates were collected from 9040 blood cultures at hospital and diagnostic center in Dhaka Division, Bangladesh. The prevalence of *Salmonella typhi* was 74.5% (785) and *S. paratyphi* A were 25.5%. The isolation rate was highest between the age group 25 to 60 months, male showed slightly higher rate of infection than female. Among all 943 *Salmonella typhi* isolates studied, 57.4% were found resistant to ampicillin, 58% found resistant to cotrimoxazol. 58.6% was resistant to chloramphenicol, 9 isolates we have collected were ciprofloxacin resistant and 97.8% were found nalidixic acid resistant. On the other hand, all isolates were sensitive to ceftriaxon and ceftazidim. In E-strip test, we have found that 95 isolates showed between 0.5 mg/ml to 2.0 mg/ml and 11 strains showed from >2.0mg/ml to very highly resistant (512mg/ml). Ciprofloxacin-resistant *S. typhi* carrying patients were treated with a third generation cephalosporin and were cured without complications. Isolates were highly resistant to ampicillin, cotrimoxazole, chloramphenicol, ciprofloxacin and nalidixic acid, and were susceptible to ceftriaxone. All three isolates were found to be identical by Api 20 E score (4404552). Digestion of PCR product (195 bp gyraseA) with Hinfl did not make any change, compared to the undigested product of the same strains, in the electrophoresis banding pattern, indicating the proesence of mutations at both the Ser-83 and ASp-87 sites of the genome *Gyr*A. In contrast, the PCR product from nalidixic and susceptible control strain cleaved at the sites, ser-83 and asp-87.

Key words: Typhoid, Multidrug resistance, Salmonella, Prevalence, Antimicrobials.

Water borne diseases represent a serious threat to public health, affecting many people and resulting in considerable economic consequences in many parts of the world. Typhoid fever is distressingly prevalent in developing countries, where it remains a major health problem (Arora *et al*., 1992). The annual global incidence of typhoid fever caused 21 650 974 illnesses and 216 510 deaths during 2000 and that paratyphoid fever caused 5 412 744 illnesses (Campbell *et al*., 2011). In 2000, typhoid fever caused an estimated 21.7 million illnesses and 217,000 deaths, and paratyphoid fever caused an estimated 5.4 million illnesses worldwide (Crump *et al*., 2004). In one study (Ivanoff *et al*., 1994), clinical illness appeared in 99% and 89% of human volunteers who had ingested respectively 109 and 108 *Salmonella typhi* cells in 45 ml of skimmed milk respectedly. In recent years, an added public health concern has been the development of antimicrobial resistant (AMR) bacteria that markedly reduce the number of antimicrobials available for effective treatment of infectious diseases in humans and animals

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(Angulo *et al*., 2004). The emergence of MDR *Salmonella* strains with resistance to fluoroquinolones and third-generation cephalosporins is severely limit the possibilities for effective treatment of human infections. *Salmonella typhi* are resistant to commonly available antibiotics, and clinical resistance to fluoroquinolones, the most effective antimicrobials for the treatment of typhoid fever, has been reported (Levine and Levine, 1994). Infection with *Salmonella typhi*, the causative organism of this disease, requires effective antimicrobial chemotherapy in order to reduce mortality (DuPont, 1993). Chloramphenicol was the "gold standard" agent for the treatment of this infection (Islam *et al*., 1993), but with the emergence of chloramphenicol-resistant strains, ampicillin and trimethoprim were considered suitable alternatives (DuPont, 1993). Since 1989, however, multidrug-resistant (MDR) *Salmonella typhi* strains that are no longer susceptible to these three first-line antibiotics have emerged (DuPont, 1993). Unlike most developed countries, Bangladesh does not have a nationwide typhoidal Salmonella surveillance program to monitor multidrug resistant Salmonella in the human population. There are a few published studies with limited data on the prevalence and characterization of Salmonella isolates from clinical samples like blood. The objective of the present study was to determine the prevalence, distribution of serovars, antimicrobial resistance profiles, and risk factors associated with Salmonella in clinical samples in Bangladesh. These updated baseline data are needed to establish an integrated and unified antimicrobial resistance surveillance system.

MATERIALS AND METHODS

Patients selection

The Studies presented in this chapter are based on the clinical research in blood Culture proven typhoid fever patients admitted to a Dhaka Shishu hospital, Popular Diagnostic Center, Kumudiny Hospital and other sites of our study throughout Banoladesh.

Culture method

The diagnosis of enteric fever was based on isolation and identification of the bacterium in cultures. *Salmonella* isolates were isolated from Blood Agar only during the first 2 weeks of illness.The liquid medium used was Trypticase Soy supplemented Peptone broth which also contain an Anticoagulant. MacConkey agar was used for isolating pure culture.

Blood culture

Drawing 5 10 ml of blood by the syringe was poured into sterile cotton plugged bottles containing liquid medium. The patient's blood was mixed with the liquid medium and allowed to flow over the agar on one of the narrow sides of bottle. The bottle was incubated in the upright position and the agar surface examined daily for colonies. Then the colonies were plated on MacConkey agar to estabilish a pure culture (Levine *et al*., 1978).

Identification

Once growth was obtained on the solid medium a typical colony was picked up and inoculated into peptone water as well as onto MacConkey agar. The tubes were then incubated at 37°C for 24hrs. The peptone broth culture was used for typical biochemical tests (Kligler Iron Agar, Motility Indole Urease and Citrate media) and identification of *S. enterica* serovar Typhi using API 20E system (bioMe´rieux, Inc., Hazelwood, MO) (Hunter and Strickland, 1984).

Identification Using Biochemical Tests

This isolates of *S. typhi* were confirmed by standard biochemical (API 20E system, BioMeriux, France) and serologic (Murex Diagnostics, Dartford, England) means. All patients included received an effective antibiotic (i.e., an antibiotic to which individual isolates of *S. typhi* were sensitive *in vitro*). Once growth is obtained on the solid medium a typical colony is picked up and inoculated into peptone water as well as on nutrient agar. The tubes are then incubated at 37°C for 2 to 4hrs. The peptone broth is checked for motility, gram stain characteristics and biochemical tests are done.

Antigenic characterization

After reisolation and confirmation of biochemical profiles, the strains were serotyped through the identification of surface antigens with somatic antisera, and flagella antigens with flagellar antisera, following the Kauffmann-White scheme. Antigenic characterization was tested by the slide agglutination technique with poly and monovalent antisera, somatic and flagellar. Identification of specific serovars was performed and presented

according to the criteria reported by Grimont and Weill (Grimont and Weill, 2007).

Antimicrobial susceptibility test

943 strains were selected for antimicrobial susceptibility monitoring regardless of serovars. These strains were analyzed by the disc diffusion method (CLSI, annually updated), and results were interpreted according to the latest CLSI edition (Wayne, 2008). Antimicrobial drugs (Oxoid, Basingstoke, UK) representative of the following antimicrobial classes were used Amphicilin (AMP), Chloramphenicol (CHL), Cotrimoxazole (CTZ), Ciprofloxacn (CIP), Ceftriaxone (CRO), Ceftazidime (CAZ) and Nalidixic Acid (NAD). The following standard strains were used to assure quality control and reliability in the results: *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 25922, *E. coli* ATCC 35218 and *Pseudomonas aeruginosa* ATCC 27853.

Agar diffusion with E-test

The E- Strip was used for determination of MIC; E-strip was impregnated with the drugs (Ampicillin, Nalidixic acid, Chloramphenicol, Cotrimoxazole). A lawn of bacteria is spread and grown on an agar plate, and the Etest strip was placed on Muller Hintn media. After 24 hours of incubation, an elliptical zone of inhibition is produced and the point at which the ellipse meets the strip gives a reading for the minimum inhibitory concentration (MIC) of the drug. The MIC breakpoints for resistance used were those recommended by the NCCLS (Jorgensen, 1993) except for fluoroquinolones. The resistance breakpoint for ciprofloxacin was chosen as >0.25 mg/L on the basis of earlier publications (Threlfall *et al*., 1997). For norfloxacin and ofloxacin a breakpoint of 0.5 mg/L was used. The term 'quinolone resistance ' is used here to indicate resistance to nalidixic acid (break point 32 mg/L) and/or to one or more of the fluoroquinolones tested. The MIC breakpoints for the fluoroquinolones used here are much lower than those recommended by NCCLS guidelines(Gotuzzo *et al*., 1991). The breakpoint value for reduced ciprofloxacin susceptibility was chosen as >2.00 mg/mL on the basis of earlier publications (Piddock *et al*., 2002) and our recent differential bactericidal concentration analyses, combined with the sequencing data of the quinolone resistance determining region (QRDR) of the gyrA gene (Kustner, 1979).

DNA extraction

DNA from pure Culture of *Salmonella typhi* on MacConkey agar plate was extracted by procedure described by Heat lysis method. Briefly, 500mL of bacterial suspension was placed on to a heat stable floater and the whole apparatus was placed onto a boiling water bath 1 or 10 minutes assuming that all the cells were swelled and busted and genomic DNA was come out. Because of using highly powerfull (partner finding) primer of gyraseA there was no problem for PCR arnplification. After PCR amplification of gyraseA we also have confirmed the PCR product by running it through 1% polyaccrylamide gel.

Amplification of gyraseA gene

The gyrFand gyrR primers were mixed with the PCR master mix along with template genomic DNA of both ciprofloxacin sensitive and ciprofloxacin resistant strains. Then the whole PCR mix was kept into the thermocycler for gyraseA gene amplification.

RFLP analysis

The mixture was incubated for 4 hours to overnight into an incubator, which was maintained at 37^0C. After incubation, the digested mixture was then mixed with 2mL of gel loading buffer and run the total sample through 3% agarose gel kept into an electrophoresis chamber.

Statistical Analysis

The susceptibility data were analysed by using the WHONET4 computer program, available from J. Stelling (WHO/EMC, 1211 Geneva 27, Switzerland). Statistical analysis was performed using the x^2 test and Fisher's exact test. *P* values of <0.05 were considered significant.

RESULTS

Blood samples were collected from Dhaka Shishu hospital, Popular Diagnostic Center, Kumudiny Hospital and other sites of our study throughout Banoladesh. A total of 1050 Salmonella isolates were identified from 9040 blood cultures.

Prevalence of Salmonella

The prevalence of *Salmonella typhi* was 74.5% (785) and *S. paratyphi* A were 25.5% (265). The isolation rate was highest between the age group 25 to 60 months, male showed slightly higher

Table 1. Reaction Mixture for gyraseA gene amplification

Reagents	Volume(mL)
Primer(gyrF and gyrR)	0.125X2
PCR master mix	15
Template DNA	1.5
DNase free water	8.25

rate of infection than female. A total of 1050 Salmonella strains were recovered from these clinical blood samples with following typhoidal serotypes: Typhi, Paratyphi A and Paratyphi B. Of the 9040 blood cultures obtained during the study period, 1050 (11.6%) yielded significant growth. Nearly three fourths of the positive blood cultures (74.5%, 785 of 1050) were S. typhi and others were mostly S. paratyphi A. The isolation rate from boys

Table 2. Biochemical tests for different Salmonella serogroups

Organic molecules	Name of isolates		
	Salmonella typhi	*Salmonella paratyphi* A	*Salmonella paratyphi* B
Glucose	A	AG	AG
Lactose	-	-	-
Sucrose	-	-	-
Mannitol	A	AG	AG
Raffinose	-	AG	AG
Dulcitol	-	AG	AG
Sorbitol	A	AG	AG
Inositol	-	-	-
Arabinose	-	-	-
Xylose	-	AG	AG
Indole	-	-	-
Methyl Red	+	+	+
Citrate	-	-	+
Gelatin	-	-	-
Urease	-	-	-
Motillity	+	+	+
Hydrogen sulphie	+	+	+

Key: A = Acid; AG = Acid + Gas; + = Positive; = Negative

Fig. 1. Colony characteristics of *S. enterica* serovar Typh on MacConkey

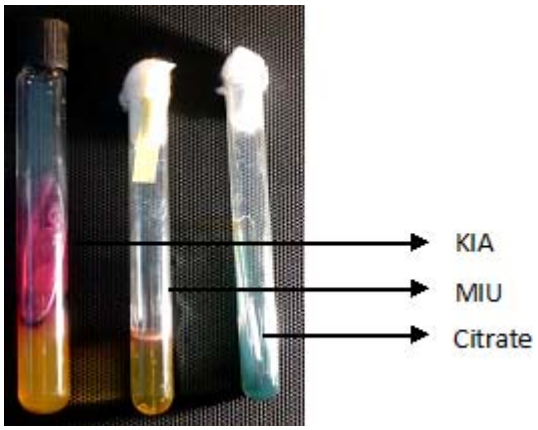


Fig. 2. Typical Biochemical characteristics of S. *enterica* serovar *typhi*

and girls was apparently similar (figure: 6.) The Isolation rate was highest bctween the age group 25 to 60 months, male showed slightly high rate of infection than female. Moreover, the isolation rate was highest in the summer and monsoon season (May to October), with peaks in September (9.7% in 2006 and 12.3% in 2007) and was relatively from November to March. These trends were also observed in the year proceeding to the study period.

Biochemical Test

Biochemical has been done for confirmation of Salmonella isolates. The results are illustrated in the (table 2) in the below

Y axis represents total number of isolates, whereas X axis represents different age groups (A] is for 0 to 24 months; A2 for 25 to 60 months,A3 for 61 to 120 months, A4 for 121 to 180 months and A5 for 191 months to maximum age of our sample collections).

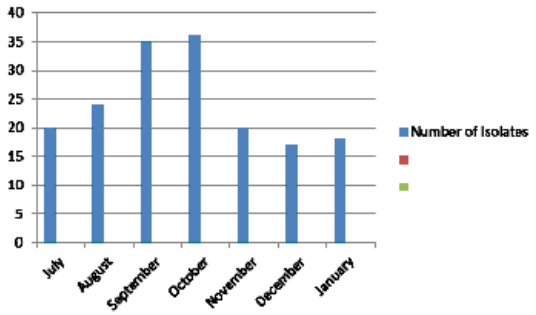


Fig. 3. Age specific Distribution of Typhoid Fever

Y axis represents total number of isolates, whereas X axis represents different age groups (A] is for 0 to 24 months; A2 for 25 to 60 months,A3 for 61 to 120 months, A4 for 121 to 180 months and A5 for 191 months to maximum age of our sample collections).

Antomicrobial Sensitivity Test

Among all 943 *Salmonella typhi* isolates studied, 57.4% were found resistant to ampicillin, 58% found resistant to cotrimoxazol. 58.6% was resistant to chloramphenicol, 9 isolates we have collected were ciprofloxacin resistant and 97.8% were found nalidixic acid resistant. On the other hand, all isolates were sensitive to ceftriaxon and ceftazidim.

Determination of MIC by E-Strip Test

Among all 943 *Salmonella typhi* isolates Studied, we have performed E strip test for 411 isolates. Of which 53 strains showed the MIC value nearer to the very sensitive (<O. 125 mg/ml), 252 numbe of strains showed between 0.125mg/ml to 0.5 mg/ml , 95 isolates showed between 0.5 mg/ml to 2.0 mg/ml and rest other 11 strains showed from >2.0 mg/ml to vary highly resistant e.g. 512mg/ml.

GyraseA gene Amplification

Primer of gyraseA and Taq DNA polymerase successfully amplified the 195 bps gyraseA gene from the genomic DNA of ciprofloxacin sensitive and ciprofloxacin resistant *Salmonella* serover *typhi*.

RFLP Analysis for Determination of Mutation position

Digestion of PCR product (195 bp gyraseA) with *Hinf* I did not make any change

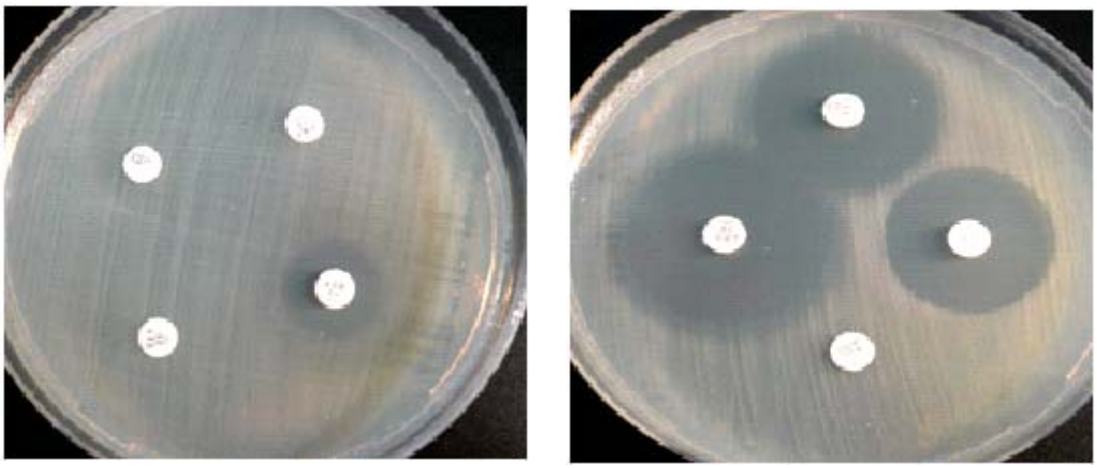


Fig. 4. Response *of Salmonella typhi to* different antibiotics

compared to the undigested product of the same strains, in the electrophoresis banding pattern,indicating the presence of mutations at both the Ser 83 and Asp 87 sites of the genome GyrA (RFLP pattern A). In contrast, the PCR product from nalidixic acid susceptible control strain cleaved at both stie ser 83 and asp 87 (RFLP pattern D). The Resulted figure is given in below:

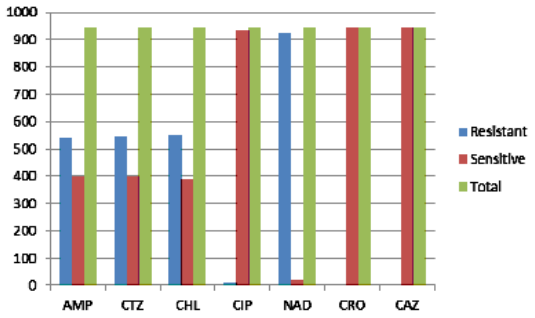


Fig. 5. Antimicrobial resistance pattern

Positive Control ($^{+}$ve) = 530 gyrase product which has two mutations at 87 and 83 codon position will not be digested after Hind restriction enzyme treatment. So the 195 bps of PCR product will be visualized on Agarose gel

Negative Control ($^{-}$ve) = 243 (RE) nalidixic acid sensitive which has no mutation at 87 and 83 codon position will be digested after Hind restriction enzyme treatment and reveals 137bps PCR product on Agarose gel

DISCUSSION

A reliable system to identify and to report the cases of typhoid fever does not exist in Bangdadesh like many developing countries where the disease is endemic. Thus only limited information is available regarding the epidemiology of typhoid fever in the community. Our study

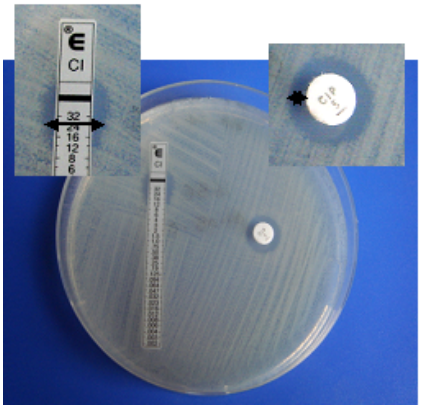


Fig. 6. E-test for CIP resistant strain

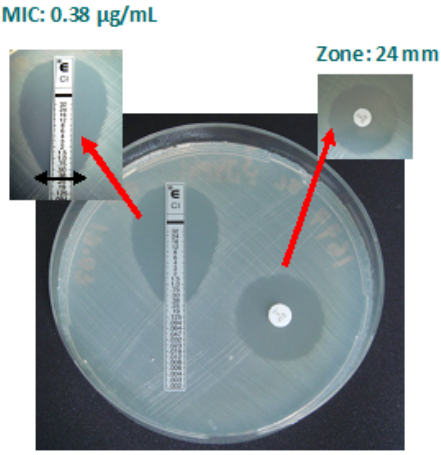


Fig. 7. E-test of CIP sensitive strain

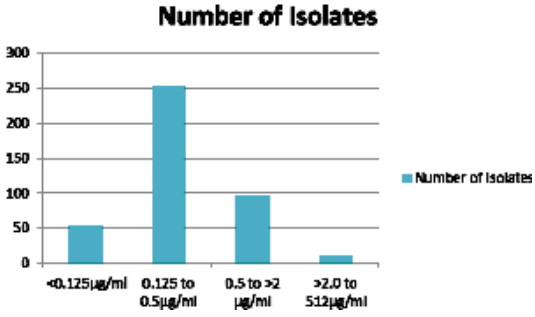
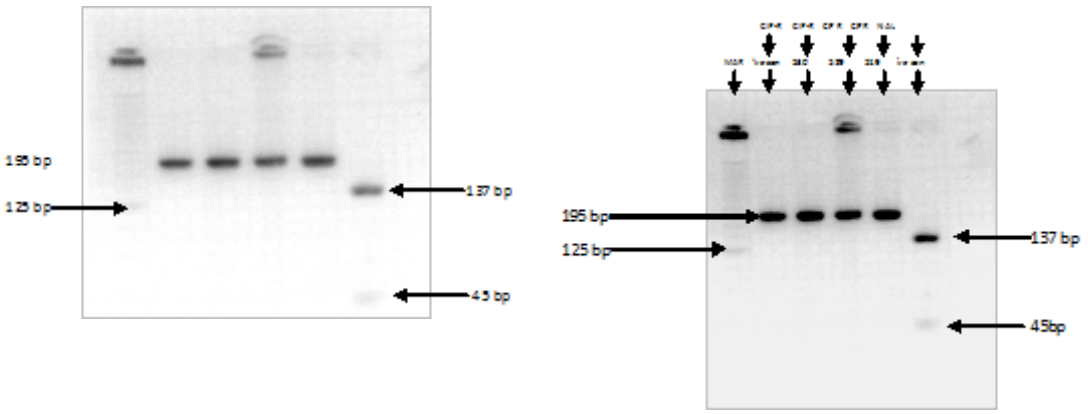


Fig. 8. E-strip test of 411 *Salmonella typhi* isolates for ciprofloxacin sensitivity

determined the prevalence, serovar distribution, and antimicrobial resistance phenotypes of Salmonella in blood cultures in Bangladesh.

There is a common belief that the disease has the highest incidence in 5 -to 12 year old children. Thus efficacy trials with either with Vi antigen vaccine or live attenuated Ty 21a vaccine were done in this age group, and vaccines are currently recommended for use in school age children and adults. The high isolation rate and degree of bacteraemia in younger children suggest that they had a greater burden of infection.



Positive Control (+ve) = 530 gyrase product which has two mutations at 87 and 83 codon position will not be digested after Hind restriction enzyme treatment. So the 195 bps of PCR product will be visualized on Agarose gel
Negative Control (-ve) = 243 (RE) nalidixic acid sensitive which has no mutation at 87 and 83 codon position will be digested after Hind restriction enzyme treatment and reveals 137bps PCR product on Agarose gel

Fig. 9. The amplified product of Ciprofloxacin resistant (high MIC) *Salmonella typhi* digested by *Hind* restriction enzyme which is specific for it

Moreover the patients, from whom blood Cultures were obtained, at this relatively expensive refferal center, were largely from the middle and upper income population of Dhaka city who have access to piped water. Thus the incidence of typhoid fever in urban slum and rural areas of Bangladesh, which do not have safe water supplies, might be even higher than our data would indicate.

Our study shows a dramatic increase in the annual proportion of reduced fluoroquinolone susceptibility from 3.9% to 23.5% among all the *Salmonella* serover *typhi* in Bangladeshi between 1999 and Jan 2007. The increasing trend was particularly notable among isolates collected from the children of age between 2 to 5 years, Moreover, 3.5% of Salmonella isolates had reduced fluoroquinolone susceptibility. The common nature of quinolone resistance in Southest Asia revealed that a tourist's risk of acquiring (quinolone resistant salmonellosis was significantly higher in Thailand and Malaysia than in other destinations. On this basis, the emergence of antimicrobial resistance in any part of the world may have a global bearing and thus deserves universal attention.

The overall Salmonella prevalence was 11.6% (1050). The prevalence of *Salmonella typhi* was 74.5% (785) and *S. paratyphi* A were 25.5%. Among all 943 *Salmonella typhi* isolates studied, 57.4% were found resistant to ampicillin, 58% found resistant to cotrimoxazol. 58.6% was resistant to chloramphenicol, 9 isolates we have collected were ciprofloxacin resistant and 97.8% were found nalidixic acid resistant.

In Bangladesh, the possibility of clonal spread as a major contributing factor was excluded by identification of 8 serotypes among the quinolone resistant isolates. In addition, some of these serotypes contained different antimicrobial resistance patterns along with their different VNTR patterns. Based on these data, we can easilv conclude that the reduced fluoroquinolone susceptibility of *Salmonella typhi* in Bangladesh primarily involves mutations in chromosomal genes. This concept is consistent with our VNTR experiment and finally proved by sequencing data: all 8 *Salmonella* isolates with reduced fluoroquinolone susceptibility so far analyzed in our laboratory have shown point mutation leading to amino acids change in their QRDR of the gyraseA gene.

Accordingly, the alarming increase in quinolone resistance observed during the past few years among foodborne pathogens (Sáenz *et al*., 2000) has aroused speculation that this might be an effect of the use of quinolones in animal husbandry (Medders *et al*., 1998). Indeed, two recent articles (Giraud *et al*., 1999) have shown that enrofloxacin (a fluoroquinolone used in agriculture) can select Salmonella mutants resistant

to nalidixic acid and fluoroquinolone. No part of the world allows quinolone to be used as growth promoters, but they have been licensed for therapeutic use in food animals in many countries. In Asia, several quinolone including three fluoroquinolone licensed for humans (ciprofloxacin, ofloxacin, and norfloxacin), have been approved for animal use (Organization, 1998). The policy is more strict in the United States, where the only quinolone licensed for food animals is enrofloxacin (McKellar *et al*., 1999), which is allowed for treatment of poultry alone (Organization, 1998). Without any data on the consumption figures of the quinolone antimicrobial group, no conclusions can be drawn on a potential link between the reduced fluoroquinolone susceptibility of *Salmonella typhi* and the use of quinolones in animal husbandry. Yet, such a connection is plausible.

In conclusion, we have shown that reduced susceptibility of salmonellae to the fluoroquinolone group was significantly associated with multi-drug resistance. Moreover, all quinolone resistant Salmonella isolates had undergone a point mutation in the QRDR of the gyrA gene. In contrast to previous reports on quinolone resistance in a specific clone or in a few *Salmonella* serotypes, the reduced fluoroquinolone susceptibility of our isolates was nonclonal. These data provide further evidence of the rapid spread of multidrug resistant pathogens from one continent to another. The ernergence of antimicrobial resistance in any part of the world may have global implications and is, therefore, of universal concern.

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