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Antibiotic resistance and presence of plasmids in clinical isolates of *Salmonella*

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Abstract: In the present study seventeen clinical isolates of *Salmonella typhi*, obtained from Children's Hospital, PIMS, Islamabad, Pakistan, during 2006 were monitored to check the level of antibiotic resistance against twelve commonly used antibiotics. Out of seventeen isolates, fifteen showed multiple resistances to four or more of the antibiotics, while two isolates were resistant to two/three antibiotics. Resistance against streptomycin and spectinomycin was expressed by all isolates. Gentacin and Ofloxacin proved to be potent antibiotics against *Salmonella typhi*. All the isolates analyzed for plasmid DNA by agarose gel electrophoresis, showed a similar profile. Seven bands were observed ranging in size from 21 kb to 1.3 kb.

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INTRODUCTION

Salmonella is a gram negative, motile, oxidase-negative, facultative bacterium (bacillus), belonging to the family *Enterobacteriaceae.* It has been divided into different subclasses, only some of them are medically important, which are *Salmonella typhi*, *S. paratyhi* A, *S. paratyphi* B, *S. paratyphi* C, *S. typhimurium, S. enteritidis* and *S. cholerae*- suis.

Salmonella which causes *salmonellosis,* are intracellular parasites responsible for a range of syndromes in vertebrates ranging from acute self-limiting gastroenteritis to typhoid[1]. They cause a wide spectrum of human diseases i.e. from self-limited watery diarrhoea to inflammatory diarrhoea and sometimes bacteremia, enteric fever and focal extra-intestinal infections[2] .

Salmonella typhi causes typhoid fever. The characteristic lesions of typhoid fever are in the lymphoid tissues of the intestinal wall and mesenteric lymph nodes. Typhoid fever may also be accompanied by disseminated intravascular coagulations leading to haemorrhage[3]. A variety of complications may occur including laryngeal ulcer, infection in gall bladder and inflammatory process in different parts of the body, disease of bone-marrow and joints.

Typhoid fever is an unsolved health problem in the world[4]. There are approximately 16 to 33 million cases globally with over 500,000 deaths in the WHO report (WHO, 2008). Ninety three percent of these cases are reported in Asia[5]. The number of human *Salmonella* isolates reported to the Centre for Disease Control, USA has been increasing since 1970. *Salmonella paratyphi* A and B cause paratyphoid fever which is like typhoid fever but not as much severe. *Salmonella paratyphi* C causes septicaemia and is uncommon cause of infections in humans[6]. Non-typhoidal salmonella are major causes of diarrhoeal disease that is gastroenteritis and bacteraemia in children[7-9].

Resistance to antimicrobials is thought to be a major worldwide problem[10-11]. *Salmonella typhi* is resistant to penicillin, but show in vitro sensitivity to several of the broad spectrum antibiotics. Chloramphenicol is the most effective of these in vitro and has for many years been the drug of choice in typhoid infections[12,13]. According to the data from Centre for Disease Control USA *salmonella* strains developed increasing resistance to multiple antibiotics between the mid – 1960's and mid 1970's. In 1979 and 1980 the most common resistance pattern was R type Cm Sm Su Tc Tp Chloramphenicol (Cm), streptomycin (Sm), sulphonamides (Su), tetracycline (Tc) and trimethoprim (Tp) but in subsequent years strains of R-type Ap Cm K Su Tc Tp (Ap-ampcillin, K-Neomycin – Kanamycin) have predominated[14,15].

The biochemical modification which cause resistance are controlled encoded at the genetic level by cell's own chromosomal DNA or it may be due to the presence of extra chromosomal DNA replicons that is the plasmids[16].

MATERIALS AND METHODS

Salmonella isolates (seventeen) were obtained from Pakistan Institute of Medical Sciences (PIMS), Islamabad. These pathogens were isolated from the stool sample of patients suffering from typhoid and paratyphoid fever. These isolates were identified in the Quaid-i-Azam University, Department of micro biology, on the basis of routine serological tests as described in the Medical laboratory manual for Tropical Countries[15].

Bacterial cultures were stored in the freezing medium and skimmed milk medium at 70°C. In routine experiments the cultures were maintained on LB-Agar slants at room temperatures and sub-cultured bimonthly.

All chemicals used in this study were of molecular biology grade and were obtained from E. Merck and Sigma Chemical Company. Culture media were purchased from Oxide limited and Difco Laboratories.

Determination of antibiotic susceptibilities

Antibiotic susceptibilities of *Salmonella* isolates were tested by the Disc Diffusion Method of **Bauer *et al.*, (1966).** The drugs tested were ampicillin (AP), chloramphenicol (Cm), tetracycline (Tc), gentamicin (Gm), kanamycin (Km), streptomycin (Sm), spectinomycin (Sp), amikaicin (AK), penicillin (Pn), novobiocin (Nb) trimethoprim (Tp) and ofloxacin (Of). The amounts of antibiotics were measured in micrograms of antibiotic per disc (µg/disc). The antibiotics were dissolved in different volumes of ethanol and water. MIC was done to test each isolate against various antibiotics. LB–Agar was used as growth medium for all susceptibility testing. Antibiotic susceptibility was also tested by using the following standard discs. Fosphomycine (FOS) (50 µg/disc), Ofloxacin (OFX) (5 µg/disc), Ceftazidime (CAZ) (50 µg/disc), Naldixique (NA) (30 µg/disc), Cefotaxime (CTX) (30 µg/disc), Enoxabid (ENX) (30 µg/disc), Cefazolin (CZ) (30 µg/disc) and Amoxicilline (AMX) (25 µg/disc).

Plasmid DNA isolation

Plasmid DNA was isolated by using the method of [26]**.** Agarose gel electrophorsis was employed to analyze the plasmid DNA[16]**.** The gels were stained in ethidium bromide (0.5µg/ml) solution and photographed using a combination of UV filter and red Kodak Wratten Gelatine filter on Kodak high speed micro film.

RESULTS AND DISCUSSION

The analysis showed that all the seventeen *Salmonella* isolates used in this study were sensitive to gentamicin and all the isolates showed resistance to spectinomycin and streptomycin. Table 1 represents the numbers of isolates resistant to individual antibiotics (µg/disc). The pattern of resistance to different antibiotics tested for individual isolates are given in Table 2. Further two isolates i.e. FI-2 and FI-13 shared multiple resistance to varying concentrations of nine of the eleven antibiotics tested. They both showed resistance against the same concentrations up to a maximum of 100 µg/disc of Ap, Sm, Nb, Tp as tested in this study while resistance was observed at 3.125 µg/disc of Km. FI-2 and FI-13 however, were resistant to Sp, Cm and Pn at different concentration levels. FI-2 levels were Sp 12.5 µg/disc and, for Cm and Pn the levels for resistance were 100 µg/disc. FI-13 showed resistance against 25 µg/disc of each for Sp, Cm and Pn. In addition FI-2 and FI-13 were resistant to Ak at 3.125 µg/disc and Tc at 25 µg/disc respectively. Seven out of seventeen isolates i.e. FI-5, FI-8, FI-9, FI-10, FI-14, FI-15 and FI-16 were resistant to eight of the eleven antibiotics at varying concentrations. The resistance pattern for FI-5, FI-8, FI-9, FI-14 and FI-16 was similar [Ap-Sp-Sm-Cm-Tc-Pn-Nb-Tp] but the antibiotic concentrations to which these were resistant were different. However, isolate number FI-10 showed the resistance pattern of [Ap-Ak-Sp-Sm-Cm-Tc-Pn-Tp] with the antibiotic concentrations of Ap, Sm, Cm, Tc, Pn, Tp 100 µg/disc, Ak 3.125 µg/disc and Sp 12.5 µg/disc. One isolate, FI-17, was found to resist six antibiotics and had a pattern of [Ap-Sp-Sm-Cm-Tc-Nb]. Isolate number FI-12 showed resistance against five antibiotics. Two of the seventeen *Salmonella* isolates i.e. FI-3 and FI-11 were resistant to four antibiotics and had a pattern of [Sp-Sm-Tc-Nb] and [Sp-Sm-Cm-Nb] respectively. The FI-6 was resistant to three antibiotics. Only one isolate i.e. FI-4 was found to resist two antibiotics [Sp-Sm], 12.5 µg/disc each, out of eleven antibiotics tested. In this way the resistance against Sp and Sm was 100% while it was 70.5% for Cm, Nb and Pn; 64.7% for Ap, Tc and Tp; 23.5% for Km and only 11.7% for Ak. **(**Tables 1 and 2)

All the seventeen *Salmonella* isolates were sensitive to the following antibiotics: CTX 30 µg/disc, NA 30 µg/disc, OFX 5 µg/disc, CAZ 30 µg/disc, ENX 30 µg/disc and CZ 30 µg/disc. In addition FI-1, FI-3, FI-4, FI-6 and FI-11 were also sensitive to AMX 25 µg/disc while all other were completely resistant to it. Except two isolates i.e. FI-6 and FI-9 all the isolates were sensitive to FOS 50 µg/disc.

Plasmid DNA profile analyses

Plasmid DNA profiles of seventeen *Salmonella* isolates are represented in Figures 1 and 2, a multiple band pattern was seen in all the seventeen strains. The plasmid DNA profiles of isolates FI-1, FI-2, FI-3, FI-4, FI-5, FI-6 FI-7, FI-8, FI-9, FI-10, FI-11 and FI-12 are shown in the Fig. I. Seven bands are present uniformly in the profiles of all the isolates. Two bands of 9.07Kb and 3.8Kb appear to be strongly stained and are of almost equal density. Two bands of approximately 21Kb and 1.3Kb molecular weights are less strongly stained as

compared to the 9.07Kb and 3.8Kb bands. In addition three bands with molecular weights of 6.0Kb, 3.0Kb and 2.7Kb are also discernable in all the profiles i.e. these are weakly stained. Close examination of the gel photograph reveals further 4 bands having molecular weights of 16Kb, 14Kb, 12Kb and 9.6Kb i.e. these are very weakly stained.

Table 1: Antibiogram of individual resistant isolates of *salmonella*

Antibiotic	No. of Isolates Resistant to Antibiotic µg/disc						Resistance (%)
	100	50	25	12.5	6.25	3.125	
Ak	-	-	-	-	-	2	11.7
Ap	10	1	-	-	-	-	64.7
Cm	9	1	2	-	-	-	70.5
Gm	-	-	-	-	-	-	Zero
Km	-	-	-	-	-	4	23.5
Nb	4	-	2	2	2	2	70.5
Pn	7	2	2	-	1	-	70.5
Sm	11	-	2	3	1	-	100
Sp	1	2	8	4	2	-	100
Tc	7	2	2	-	-	-	64.7
Tp	11	-	-	-	-	-	64.7

The number 1,2,3,4, etc., represent the antibiotic concentration (µg/disc).1 = 100 µg/disc, 2 = 50 µg/disc, 3 = 25 µg/disc, 5 = 6.25 µg/disc, 4 = 12.5 µg/disc, 6 = 13.125 µg/disc.

Figure 1: Agarose gel electrophoresis (0.8%) of plasmid DNA of *Salmonella*.Distribution of samples in each lane is as follows: Lane–A (X-Hind III); Lane-1 (FI-1); Lane-3 (FI-3); Lane4 (FI-4); Lane5 (FI-5); Lane-6 (FI-6); Lane-7 (FI-7); Lane8 (FI-8); Lane9 (FI-9); Lane-10 (FI-10); Lane-11 (FI-11); Lane12 (FI-12).

The Figure 2 shows the plasmid DNA profiles of isolates FI-13, FI-14, FI-15, FI-16 and FI-17. The plasmid profiles for these five isolates are similar. There are two strongly stained bands of molecular weights 9.07 Kb and 3.8 Kb. Two other prominent bands of molecular weights 21 Kb and 1.3 Kb are also present. But these bands are less strong as can be seen by staining. In all these isolates three weakly stained bands of approximate molecular weights of 6.0 Kb, 3.0 Kb and 2.7 Kb are also common. In addition three bands of very low intensity and molecular weights of 16 Kb, 14 Kb, and 12 Kb can also be observed. A very weak 9.6 Kb band is present in all the isolates.

Resistance in bacteria to commonly used antibiotics has become a problem in clinical medicine. The extensive use of antibiotics has resulted in the emergence of resistances in clinically important bacteria. The data obtained from the analysis of *Salmonella* isolates in this study indicate a high incidence of multiple resistances. These results reflect the overall pattern and levels of antibiotic resistances in the existing *Salmonella* populations. All the isolates analysed showed multiple resistances towards four or more antibiotics with the exception of two isolates which were resistant towards two/three antibiotics. From the analyses of these representative isolates it can be concluded that gentamicin and tarivid are the most effective antibiotics, since all the isolates were sensitive to these even at a very low concentration i.e. 3.125 µg/disc and 5 µg/disc respectively.

Table 2: Levels of resistances for individual antibiotics among the seventeen isolates of *salmonella.*

Isolate No.	Antibiotic Resistance Phenotype
FI-2	Ap^1-AK^6-Sp^4-Sm^1-Cm^1-Pn^1-Nb^1-Km^6-Tp^1
FI-13	Ap^1-Sp^3-Sm^1-Cm^3-Tc^3- Pn^3-Nb^1-Km^6-Tp^1
FI-5	Ap^1-Sp^1-Sm^1-Cm^1-Tc^2- Pn^1-Nb^5-Tp^1
FI-8	Ap^1-Sp^3-Sm^1-Cm^1-Tc^1- Pn^1-Nb^5-Tp^1
FI-9	Ap^1-Sp^3-Sm^1-Cm^1-Tc^1- Pn^1-Nb^1-Tp^1
FI-10	Ap^1-Ak^6- Sp^4-Sm^1-Cm^1-Tc^1- Pn^1-Tp^1
FI-14	Ap^1-Sp^5-Sm^1-Cm^2-Tc^2- Pn^1-Nb^3-Tp^1
FI-15	Ap^2-Sp^5-Sm^3-Cm^1-Tc^1- Pn^2-Nb^4-Tp^1
FI-16	Ap^1-Sp^2-Sm^1-Cm^1-Tc^1- Pn^2-Nb^6-Tp^1
FI-7	Ap^1-Sp^4-Sm^1-Cm^1-Tc^1- Pn^1-Tp^1
FI-17	Ap^1-Sp^2-Sm^1-Cm^1-Tc^1-Nb^6
FI-12	Sp^3-Sm^3-Pn^3-Km^6-Tp^1
FI-1	Sp^3-Sm^4-Nb^6-Km^6
FI-3	Sp^3-Sm^5-Tc^3-Nb^3
FI-11	Sp^3-Sm^1-Cm^3-Nb^5
FI-6	Sp^3-Sm^4-Pn^5
FI-4	Sp^4-Sm^4

Plasmid DNA Pattern analysis is now routinely used for characterizing the gram-positive as well as gram negative organism[17-22]**.** Such an approach has also been used to find out the causative agent in outbreaks of enteric diseases such as *Salmonellosis* [3, 8, 9, 23, 28, and 36]. This technique compares

favourably with bacteriophage typing[23], biotyping and antibiotic resistance pattern[23-26]. It is accepted as a means of identifying relatedness or unrelated-ness of strains of *Salmonella*[27-30].

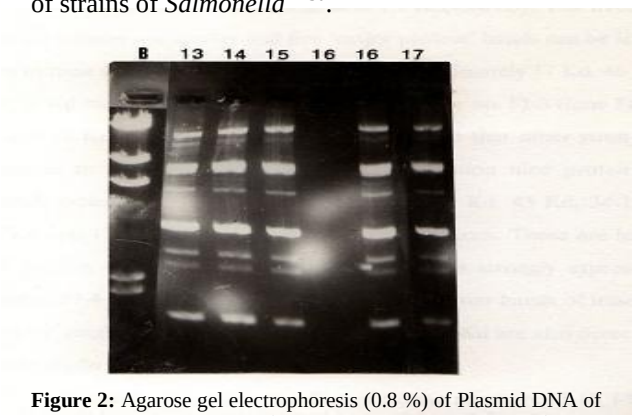

Figure 2: Agarose gel electrophoresis (0.8 %) of Plasmid DNA of *Salmonella*. Distribution of samples in each lane is as follow: Lane-B (X-Hind III); Lane-13 (FI- 13); Lane-14(FI-14); Lane-15(FI-15); Lane-16 (FI-16); Lane-17 (FI-17)

In the present study all the *Salmonella* isolates have similar plasmid profiles. There are seven prominent plasmid DNA bands of 21 Kb, 9.07 Kb, 6 Kb, 3.8 Kb, 3 Kb, 2.7 Kb and 1.3 Kb molecular weight. On the basis of similarity in the plasmid DNA profiles all the isolates can be placed in one group. Previous reports show that salmonella spp. usually harbour a heterogenous population of plasmid, which has been cited as encoding virulence function[33-35].

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