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Research Article

Emerging of Multiple Drug Resistance in *Salmonella typhimurium* Serovars Isolated from Indian Ganga River

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ABSTRACT

Salmonella is an important pathogen responsible for food borne illness and other enteric diseases in human and animals. Development of drug resistance in these microbes leads to failure of therapeutic attempts and posing the public health risk. Present study was undertaken to observe the drug resistanance profile of *Salmonella typhimurium* isolated from different locations of Indian Ganga river. A total of 44 *salmonella* isolates including 24 *S. typhimurium* serovars were subjected to antibiogram test by disc diffusion method. The plasmid was isolated and compared with drug resistance pattern to describe its role in resistance mediation. All *S. typhimurium* serovars were resistant to ampicillin (100%) followed by tetracycline (91.6%), cephatoxime (75%) and nitrofurantoin (66.6%). A few isolates (6.8%) showed resistant to even ciprofloxacin. Gentamicin was the only antibiotic that showed 100% sensitivity to all isolates. Though these isolates showed resistance to most of the commonly used antibiotics, plasmid mediation of drug resistance was not observed. Our study indicates the presence of pathogenic *Salmonella* serovars in Indian water body and their increasing percentage of multiple drung resistance which is not solely mediated by plasmids. ll

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Some of the newly –emerging resistant bacteria are transmitted to human population mainly via food and water. The best-known examples are the food borne pathogens like *Salmonella* and *Campylobacter* and commensal *Enterococcus*. Some *Salmonella* serovars are more pathogenic and common bacterial causes of food-borne diarrhoea complaint worldwide (Becker et al., 2013). Though the infection by these organisms can be treated easily using commonly available antibiotics, development of drug resistant strains become common phenomena now a day due to indiscriminate use of antibiotics at sub-therapeutic concentrations and as feed supplements and growth promoters in animal husbandry practices. These practices are often associated with the development of resistance to multiple numbers of antibiotics. Multiple drug-resistant (MDR) strains of *Salmonella* are now encountered frequently and the rate of multidrug-resistance development has increased considerably in recent years (Mijovic et al., 2012). Emerging of multiple drug resistancy among several *Salmonella* serovars including *typhimurium*, *newport* and other serovars noticed wordwide (Barraw *et al*., 1988; Zhao *et al*, 2003; Li *et al*., 2012; Hannan *et al*., 2013; Van Kessel *et al*., 2013 Even worse, some variants of *Salmonella* has developed multiple drug-resistances as an integral part of the genetic material of the organism, therefore likely to retain their drug-resistant genes even when antimicrobial drugs are no longer used. The emergence of MDR *Salmonella* strains with resistance to fluoroquinolones and third-generation cephalosporins (Bhattacharya *et al*., 2011) is a serious issue, which results in severe limitation of the possibilities for effective treatment of human and animal infections. Hence, an attempt has been made to understand drug sensitivity pattern of these *Salmonella* isolates and the role of plasmids in resistance mediation among the isolates of Indian Ganga river.

Salmonella serovars (isolated from Indian Ganga river water at different places and serotyped at National *Salmonella* Centre, Indian Veterinary Research Institute, Izatnagar, India) were obtained from Animal Biotechnology Centre, College of Veterinary and Animal Sciences, Govind Ballabh Pant University of Agriculture and Technology, Pantnagar, India. Stock cultures were revived by streaking on MaConkey Lactose Agar (MLA) and Brilliant Green Agar (BGA) and observed the colony characteristics specific to *Salmonella* genus. Biochemical tests were performed to confirm the *Salmonella* using Hi*Salmonella*™ Identification kit (Himedia, Mumbai, India). In total, 44 isolates of *Salmonella* were used in the study which includes *S. typhimurium (24)* , *S. abuja*, *S. lagos*, *S. pontypridd*, *S. oritamerin*, *S. chinkual*, *S. goldenberg and S. zwickau* (isolated from different locations of ganga river bank including Haridwar, Hastinaupur, Gurmukteshwar and Narora). Out of 44 isolates characterized, 24 isolates of *S. typhimurium* tested for its antibiotic sensitivity due to its common occurance as food borne pathogen.

Antibiotic sensitivity test was performed according to Kirby–Bauer method (Bauer *et al*., 1966) Sterile Muller Hinton Agar plates were prepared and incubated for overnight at 37°C

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for testing its sterility. Two mL of overnight grown culture was spread over the surface of the agar plate and allowed to absorb as described. Antibiotic discs (Himedia, Mumbai, India) used were as follows; Ciprofloxacin (5µg), Ofloxacin (5 µg), Norfloxacin (10 µg), Nitrofurantoin (300 µg), Cotrimaxazole (25 µg), Cephotaxime (30 µg), Cephalexin (30 µg), Chloramphenicol (30 µg), Nalidixic acid (30 µg), Oxytetracycline (30 µg), Ampicillin (10 µg), Gentamicin (10 µg), Streptomycin (10 µg), and Tetracycline (30 µg).The antibiotic discs were placed properly at equal distance with the help of sterile forceps. Plates were incubated for 24 hours at 37°C and the zone of inhibition for each disc was measured in millimeter (mm) and compared with respective standard minimum inhibitory concentration (MIC) to interpret the results of test culture as resistant (R) or sensitive (S).

From the overnight grown culture, plasmid isolation was carried out as per the manufacturer protocol (QIAGEN, USA). Briefly, 50 mL of overnight grown culture was pelleted by centrifugation and 4 mL of buffer P1 was added and mixed. Then 4 mL of buffer P2 was added and mixed by inverting and kept at room temperature for 5 minutes followed by addition of 4 mL of P3.. Lysate was transferred to the QIAfilter (provided with kit) cartridge and kept at room temperature for 10 minutes. Meanwhile QIAGEN–tip 100 was equilibrated with 10 mL of buffer QBT and the content of catridge was plunged in to the tip. After complete emptying, plasmid DNA was eluted with 5 mL of buffer QF and precipitated by adding 3.5 mL of isopropanol. Then it was dried and resuspended in 50 µL of TE buffer (pH^{P} 8.0). The size of the plasmids was determined by gel electrophoresis (1% agarose) run along the ruler plasmid (*E.coli*, MTCCV517)

Table 1: Different drug resistant pattern of *Salmonella typhimurium* isolates.

Resistant to no. of antibiotics	Drug resistance pattern (no. of isolates)	Isolate	Presence of plasmid	
			Large plasmid	Small plasmid
11	Cf, Of,Nx,Nf,Co,O,Ce,Cp, A,T,S.(1)	G–20	1	–
7	Nf,Co,O,Ce,A,T,S.(1)	G–23.	–	4
6	Nf,O,Ce,A,T,S.(3)	G–10	1	–
		G–33	1	–
		G–50	–	–
	Co,O,Ce,A,T,S.(1)	G–21	–	1
	Nf,O,Ce,Cp,A,T (1)	G–38	1	–
5	Nf,O,Ce,A,T (4)	G–14	1	–
		G–37	1	–
		G–44	–	–
		G–53	1	–
	O,Ce,Cp,A,T (1)	G–28	1	–
	Nf,O,A,T,S (1)	G–35	–	–
	Nf,Co,Ce,A.S (1)	G–36	1	2
4	O,Ce,A,T (2)	G–45	1	–
		G–52	1	–
	Nf,Ce,A,T (2)	G–46	1	–
		G–55	1	–
3	Nf,A,T (2)	G–11	1	–
		G–47	1	–
	O,Ce,A (1)	G–29	1	–
	O,A,T (1)	G–40	1	–
2	A,T (2)	G–1	–	–
		G–16	–	–

Cf-ciprofloxacin, O-ofloxacin, Nx-norfloxacin,Nf-nitrofurantoin, Co-cotrimaxazole,Ce-cephotaxime, Cp-cephalexin, O-oxytetracycline,A-ampicillin, G-gentamicin, S-streptomycin and T-tetracycline.

Among these 24 isolates of *S. typhimurium*, G–20 and G–39 showed resistance to all except Chloramphenicol and Gentamicin, Norfloxacin and Gentamicin respectively. Isolate G–19 showed sensitivity to only 4 antibiotics (Nx, Cp, S, G). Remaining all the isolates showed resistance to more than two antibiotics. A total of 15 types of drug resistance pattern were observed in *S. typhimurium* (indicated in Table 1. Gentamicin was the only antibiotic found to be sensitive to all the isolates followed by Norfloxacin which was resistant to isolate G–20 only. *S. typhimurium* showed 100% resistance to ampicillin, 91.6% to tetracycline, 75% to cephotaxime, 70.8% to Oxytetracycline, 66.6% to nitrofurantoin and 33.3% to streptomycin.

Among 24 *Salmonella typhimurium* isolates, 17 isolates shown the presence of 30MDa size plasmid. Two isolates namely G–23 and G–36 had shown presence of four (16 MDa, 8.6 MDa, 4.2 MDa and 3.2 MDa) and two (16MDa and 3.9 MDa) small plasmids respectively apart from large plasmids. Remaining isolates did not show presence of any plasmids. Presence of large as well as small plasmids of isolates compared with respective drug resistance pattern. Presence of neither large nor small plasmid positively correlated with its drug resistant pattern.

Presence of typhoidal and non–typhoidal *Salmonella* with multiple drug resistance from the river water indicate the risk of people living on the river bank. Multiple drug resistance against more than 6–8 antibiotics (Cf, Of, Nx, Nf, Co, Ce, Cp, C, O, A, G, S and T) in *S. typhimurium* has been reported earlier (Saxena et al., 2004; Chiu et al., 2006 and Li et al., 2012) and it is mediated by large size plasmids (Barrow et al., 1988; Diaz et al., 2006; Chain et al., 2012). Present study shows 100 % resistance to ampicillin which is in agreement with previous studies in India (Tankhiwale et al., 2003). Though previous report says most of the *Salmonella* strains were sensitive to chloramphenicol and ciprofloxacin (Chande et al., 2002) here

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serious issue is that three isolates (6.8%) showed resistance to even ciprofloxacin. Gentamicin was the only antibiotic to which all the isolates (100%) were sensitive followed by norfloxacin (97.72%), chloramphenicol and ofloxacin (93.18%). Recent drug resistance study in India showed ciprofloxacin resistance in *Salmonella* isolates less than 2 percent and chloramphenical resistance upto 9% (Bhattacharya et al., 2011). The results of our study indicate the increasing of ciprofloxacin resistance in *Salmonella* isolates but more or less same to chloramphenicol. The acquisition of quinolone resistance is mainly due to chromosomal mutations, although a plasmid mediating quinolones resistance described (Kawakara et al., 1988). Resistance to fluoroquinolones is acquired in stepwise fashion with the introduction of chromosomal mutations that alter the target proteins either DNA gyrase as well as topoisomerase IV, or it may lessen intracellular drug accumulation by active drug efflux. Recent days, plasmid mediated low level of quinolone resistance observed in *Klebsiella pneumonia* (Li et al., 2012) and *E.coli* (mirmomeni et al., 2007). Interestingly in our observations, the isolates showing resistance to ciprofloxacin (G–20 and G–38) also having large sized plasmid but mediation of resistance by these plasmids has to be proved. Our study showed the maximum sensitivity to gentamicin (100 %) and maximum resistance to ampicillin followed by nitrofurantoin where as in earlier reports maximum resistance was observed against nitrofurazone while gentamicin was the most effective antibiotic and reported 83.33 % sensitivity to gentamicin and chloramphenicol (Nath et al., 2000; Zhao et al., 2003).

When comparing the plasmid profile with the multiple drug resistance no correlation could be made because resistance to most of the common antibiotics has been observed in both the isolates, i.e., harboring the plasmids and isolates without plasmids. For example 100 % resistance was observed to ampicillin in all isolates of S. *typhimurium*. Similar pattern was observed in case of tetracycline also as all the isolates without plasmid were not 100% sensitive to this drug and all with plasmid did not show 100% resistance. MDR in these isolates not solely depend on the presence of plasmids of neither large size nor small size but other factors like chromosomal mutations, physiological adaptations also need to be considered to characterize the MDR completely. Plasmid mediated ciprofloxacin resistance in two isolates has to be investigated further in more number of isolates before coming to a conclusion. Regarding therapeutic point of view, though the use of quinolones is a common practice and resistant to these drugs is keep on increasing and now the time to rethink about alternate antibiotics apart from highly sensitive gentamicin. It is necessary to take some precautions like restricted antibiotic use in the treatment of human beings as well as animal husbandry practices to control increasing frontline antibiotic resistance. From these observations, we can conclude that ciprofloxacin resistance in *Salmonella* is emerging and resistance to most commonly used antibiotics is increasing in Indian continent.

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