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RESEARCH ARTICLE

STUDY OF CONJUGATE PLASMID AMONG ANTIBIOTIC RESISTANT OF BACTERIA ISOLATED FROM TYPHOID FEVER

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ABSTRACT

Typhoid fever is one of the major problems in India due to the emergence of MDR strains of *Salmonella* spp. Blood samples collected from 252 presumptive typhoid fever patients from different localities of Allahabad region were tested by Widal test and then cultured for *Salmonella* species followed by identification using standard procedures. Susceptibility pattern of the isolates against 16 antibiotics were determined by Kirby Bauer disc diffusion technique. Conjugative experiment was carried out with selected multidrug resistant isolates of *Salmonella* species.A total of 30.53% samples were positive by culture method in contrast to 66.26% blood samples tested positive by Widal test. The isolates were identified as *S. typhi* (52.94%), *S. paratyphi* A (27.45%), *S. typhimurium* (15.68%) and *S. bongori* (3.92%). Majority of isolates showed resistance to Tetracycline, Ampicillin, Nitrofurantion, Amoxicillin, Amoxicillin and Clavulanic acid while all the isolates were found resistant to Kanamycin. The isolates showed maximum sensitivity towards Streptomycin, Ciprofloxacin, Gentamicin and Ofloxacin. The multidrug resistant isolates demonstrated various drug resistance patterns. Conjugation studies showed transfer of resistance pattern (ACCoT) for majority of *Salmonella* isolates tested. Changing drug sensitivity pattern suggests need for continuous evaluation of sensitivity and resistance pattern of *Salmonella* isolates so as to make rational use of antibiotics in the management of enteric fever cases in future.

INTRODUCTION

Typhoid fever also known as typhoid is a common bacterial disease occurring worldwide transmitted by the ingestion of food or water contaminated with the feaces of an infected person (Giannella, 1996).WHO reports incidence of 22 million new cases every year of which 5% are fatal (Crump *et al.,* 2004).Until mid-1970s Chloramphenicol was the most preferable drug of choice for typhoid fever responsible for marked reduction in mortality from 10% to < 2% in the developed countries (Rowe *et al.,* 1997). Crump and Mintz (2010) reported the resistance towards the traditional first-line antibiotics such as ampicillin, chloramphenicol and trimethoprim-sulfamethoxazole defining multidrug resistance (MDR) property of *Salmonella enterica*. The spread of multi-drug resistance against chloramphenicol, ampicillin and trimethoprim-sulphamethozole caused therapeutic and public health problems in the African continent, South East Asia and Middle East since 1987 (Well, 2003). In *Salmonella enterica* serovar Typhi, R-plasmid-encoded resistance to ampicillin, chloramphenicol, cotrimoxazole and tetracycline is transferable (Jevanand *et al.,* 1997). The antibiotic-sensitive bacteria can acquire resistance traits from antibiotic-resistant strains belonging to same or different genera becoming resistant to one or more antibiotics. By studying the plasmid profile, demonstrated the transfer of drug resistance from the intestinal flora, *viz*, *E. coli* and to *Salmonella* species *in vitro* conjugation experiments demonstrated transfer of drug resistance between intestinal MDR *E. coli* and *Salmonella* species isolates. Keeping the above view in mind a study of conjugate plasmid among antibiotic resistant of bacteria isolated from typhoid fever was carried out.

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MATERIALS AND METHODS

Clinical Isolates

A total of 252 samples of peripheral blood positive for Widal test (Rapid slide test) were collected from patients attending OPD at local hospitals in Allahabad, India.

Blood Culture

An aliquot of 1 ml was inoculated in Brain Heart Infusion and incubated at 37˚C for 24 h. Positive broths were sub cultured on *S-S* Agar and MacConkey's Agar and kept overnight at 37˚C. The colonies showing typical characters were isolated for morphological and biochemical identification (Ewing *et al.,* 1986). The second portion of the sample was used for serological identification by the Widal test.

Biochemical Identification

The bacterial colonies on inoculated plates were identified on the basis of cultural, morphological and biochemical characteristics as described in Bergey's Manual of Systematic Bacteriology (Brenner *et al.,* 2005) .Colony characteristics like colony size, type of margin, elevation, texture and color of the colonies on *Salmonella Shigella* Agar and MacConkey's Agar were recorded. The isolates were following tests for identification like IMViC test, Urease test, Catalase Production, Motility test, Gelatin liquefaction, Triple sugar, Carbohydrate Fermentation and Decarboxylase test**.**

Molecular Identification

The isolates identified on the basis of morphological and biochemical characteristics were further confirmed by molecular screening (Massi *et al.,* 2005).

Isolation of Genomic DNA

Genomic DNA from the cultured bacteria in 200 µl of Tris-EDTA (TE) buffer for PCR was extracted according to the protocol's instructions using a GeneiUltrapure™ Bacterial Genomic DNA Purification kit (HiMedia Labs Mumbai). The purity of the extracted DNA was checked by measurement of A260 and by agarose gel electrophoresis.

The PCR mix Composition

The bacterial DNA extract and control was amplified using 0.5 µM primers 16S rDNA fragment. The PCR mixture (50 µl) contained bacterial DNA, PCR buffer [10 mM Tris/HCl (pH 8.3), 50 mM KCl, 3 mM MgCl, and 0.01% gelatin], 200 µM of each dNTP, and 1.0 U AmpliTaq Gold enzyme. The mixtures were amplified using *Taq* DNA polymerase. for 35 cycles at 94ºC for 1 minute, 55ºC for 1 minute, and 72ºC for 2 minutes with a final extension at 72ºC for 10 minutes in an automated thermal cycler. An aliquot of 10 µl of each amplified product was electrophorese in 2% (wt/vol) agarose gel, with a DNA Molecular Weight Marker (StepUp™500bp DNA ladder) in parallel (Massi *et al.,* 2005).

DNA Sequencing

The PCR product was sequenced using the forward, reverse and internal primer. Sequence data was aligned and analyzed with the filter option for *Salmonella* in the nucleotide BLASTN programme (Altschul *et al.,* 1990). The PCR products were loaded on 1.0% agarose gel along with StepUp™500bp DNA ladder.

Antibiotic Susceptibility Test

The antibiotic susceptibility pattern was performed using Kirby Bauer disc diffusion technique according to the guidelines provided by Clinical and Laboratory standards Institute CLSI. Overnight broth cultures (0.5 Macfarland turbidity standards) of the isolates were swabbed on Muller-Hinton Agar plates and following antibiotic discs of HIMEDIA laboratories (Mumbai, India) were impregnated after 15 minutes and incubated at 37 ±1°C for 48 h and the zones of inhibition were compared with the CLSI standards and the sensitivity pattern was determined for the following antibiotics: Ampicillin (10 µg), Amoxicillin + Clavulanic acid(30 µg), Amoxicillin (30 mcg), Azithromycin (15 µg), Chloramphenicol (30 µg), Ciprofloxacin (5 µg), Ceftriaxone (30 µg), Gentamicin (10 g), Kanamycin (30 µg), Nalidixic acid (30 µg), Norfloxacin (10 µg), Nitrofurantoin (300 mcg), Ofloxacin (5 mcg), Streptomycin (10 µg), Tetracycline (30 µg) and Trimethoprim/Sulphamethoxazole Co (1.25/23.75 µg) (Wayne, 2006).

Minimum Inhibitory Concentration

The MIC of the four antibiotics: Ampicillin, Chloramphenicol, Cotrimoxazole and Tetracycline were determined using broth microdilution technique in nutrient broth with two folds serial dilutions of antibiotics with varying concentrations of 8000, 4000, 2000, 1000, 500, 250, 120, 60 and 30µg/ml. Isolates were inoculated on to the medium and then incubated for 18-24h. Observed for turbiditylowest concentration which completely inhibits growth was considered MIC (Mandal *et al.,* 2003).

Isolation of Plasmid DNA

Plasmid DNA was isolated using alkaline lysis method. An aliquot of 1.5ml of overnight broth culture (LB Broth) was taken in an eppendorf tube and centrifuged at 6000 rpm for 2 minutes at 4˚C. The supernatant was discarded and 100µl of ice-cold Alkaline Lysis Solution–Iwas addedto the pellet which was then vortexed. Next 100µl of freshly prepared Alkaline Lysis Solution– II was added to the bacterial suspension and incubated in ice for 10 minutes. This was followed by addition of 150µl ice-cold Alkaline Lysis Solution–III to the bacterial suspension and incubation for 10 minutes in ice. The solution was then centrifuged at 12000 rpm for 10 minutes at 4˚C and then the supernatant transferred to a fresh tube. Chilled isopropanol in the ratio 0.6 volume/volume was added and then stored at -20˚C for 30 minutes. The solution was centrifuged at 12000 rpm for 20 minutes at 4˚C, and the pellet was allowed to air dry. TE buffer (150 ml) was added for dissolving the plasmid and stored at 4˚C till further use (Kado and Liu, 1981).

Agarose Gel Electrophoresis

Fifty ml of 0.8% agarose solution with 0.4gm agarose was prepared and 50ml of 1X TAE Buffer was added to it. Melting of agarose was carried out by boiling in microwave until the solution became clear followed by coolness at 65˚ C. Etbr (Ethidium bromide) at a concentration of 0.5µg/ml or (4µl) was then added to this clear solution. The solution was then poured on gel casting tray fitted with comb and left undisturbed for 20 minutes at room temperature to undergo solidification. 1X TAE

Buffer was filled in the electrophoresis unit and the casting tray was along with the gel placed in the electrophoresis unit by slight tilting. DNA samples were prepared by adding 2µl loading dye for over 5µl of DNA sample. The samples were then loaded into the wells with constant voltage (150V) supply. The mobility of sample was tracked by the movement of bromophenol blue dye and the process continued till the dye reach 3/4th of the gel length. Examination of the gel under U.V. light (transilluminator/ U.V. torch) was carried out. The plasmid was isolated (Fig. 4.8) and identified by marker plasmid DNA as described by (Datta *et al.,* 1971).

Conjugative Ability

Transfer of antibiotic resistance of the MAR bacterial strains was carried out by conjugation experiments following the standard protocols (Jevanand *et al.,* 1997). Isolates of *Salmonella* resistant to four antibiotics (Ampicillin, Chloramphenicol, Tetracycline, Cotrimoxazole) and sensitive to Nalidixic acid were selected and used as donors of R-plasmid, *Escherichia coli* 0027lac^{-}made resistant to Nalidixic acid was used as recipient. The minimum inhibitory concentrations (MIC) of the test antibiotics against the resistant *Salmonella* isolates and sensitive *E. coli* were determined before and after conjugative.Nutrient broth (Himedia) used for conjugation and solid medium employed was MacConkey's lactose agar without bile salts. Equal volumes of 6 h broth culture of donor and recipient strains were mixed and incubated overnight at 37˚ C in a water bath and plated on to MacConkey's medium without bile salts containing Nalidixic acid and one each of the various antibiotics to which the donor strain was resistant. The plates were incubated at 37˚ C for 24-48 h and examined for transconjugants. Donor and recipient strains were also inoculated individually on the media to serve as controls.The transconjugants selected were subcultured on MacConkey's agar plates and antibiotic sensitivity pattern was again checked to confirm the acquisition of R- factor (Mandal *et al.,* 2003).

RESULTS

Identification of *Salmonella* spp.

Of the 252 blood samples 167 (66. 26%) tested positive by Widal test and 51 of 167 (30.53%) were culture positive for *Salmonella*.On the basis of the morphological and biochemical characteristics of the colonies obtained on S-S agar and MacConkey agar, four *Salmonella* species were identified *viz. S. typhi* 52.94%, *S. paratyphi* A 27.45%, *S. typhimurium* 15.68% and *S. bongori* 3.92%. One isolate each from the *Salmonella* spp. identified on the basis of cultural, morphological and biochemical characteristics were further identified according to there molecular weight in base pairs. The amplified PCR products carried out using universal bacterial 16S rRNA primers and visualized by UV illumination showed the expected bands of about 1500 bp (Fig. 1). The results demonstrated correct genus identification of examined *Salmonella* isolates.

Antibiotic Susceptibility Pattern of the Isolates

Antibiotic susceptibility pattern of *Salmonella* spp. revealed majority of the isolates to be resistant to Ampicillin (92.16%), Amoxicillin (78.43%), Amoxicillin + Clavulanic acid (68.63%) and Nitrofurantoin (88.24%). All the isolates were found resistant against Kanamycin but sensitive towards Ciprofloxacin, Ofloxacin, Gentamicin, and Streptomycin. For the remaining antibiotics the isolates showed a varying degree of sensitivity pattern (Table 1). Of the 51 isolates culture positive for the test pathogen majority showed resistance to ACT (47.06%) followed by ACCo (39.21%) and ACCi (33.33%). Besides, resistance to ACNa, ACCoT and ACNaCi combination of antibiotics were also observed (Table 2). Plasmid DNA isolation of *Salmonella* isolates showing characteristic multidrug resistance pattern revealed the presence of common band of 600 bp (Fig. 2).

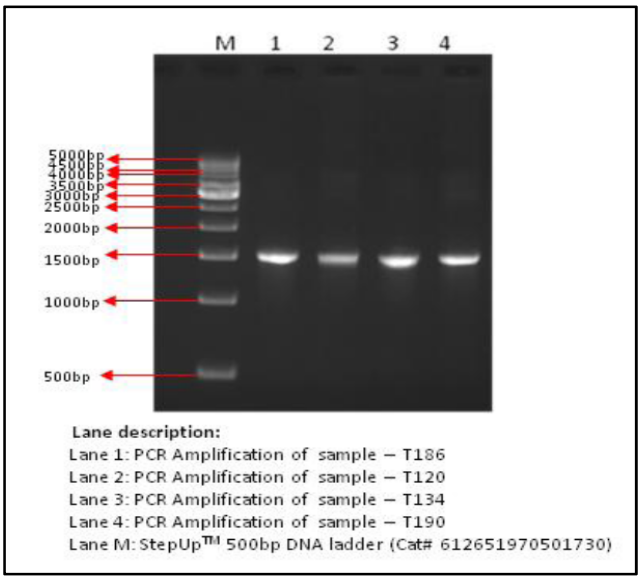

Fig. 1. Gel electrophoresis of PCR amplification

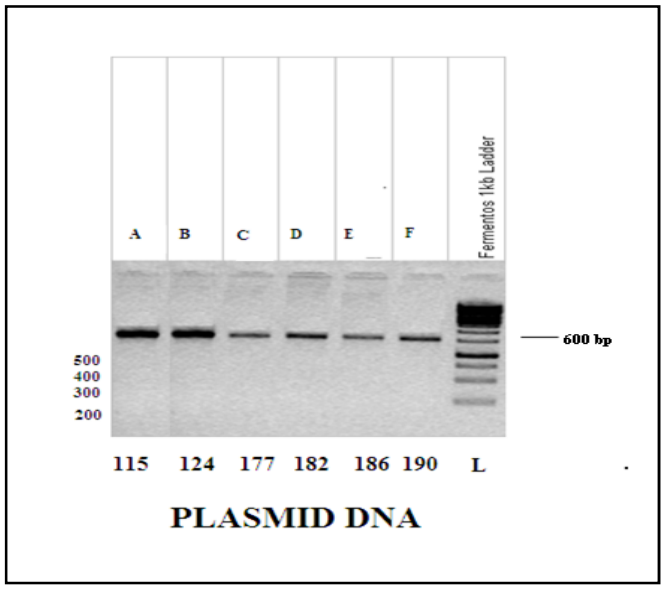

Fig. 2. Gel electrophoresis of plasmid DNA isolates from multiple antibiotic resistance *Salmonella* spp.)

Transfer of Antibiotic Resistance

In conjugation experiment four *Salmonella* isolates sensitive to Nalidixic acid and resistant to ACCoT were examined for

transfer of R-plasmids to *E. coli* which was susceptible to all drugs except Nalidixic acid. Complete transfer of resistance pattern from donor to recipient was observed for 3 isolates while 50% transfer was recorded in isolate no. 186 (Table 3).

For majority of the isolates tested MIC of test antibiotics was similar before and after conjugation studies. However, since there was no transfer of R-plasmid for Ampicillin and Cotrimoxazole from donor strain (S186) to *E. coli,* the latter remained sensitive to the drugs (Table 4).

Table 1. Antibiotic susceptibility pattern of the *Salmonella* isolates

No.	Antimicrobial agent	Disc Cons.(µg)	Resistance	Intermediate	Sensitive
1.	Ampicillin (A)	10	47 (92.16)	4 (7.84)	0 (0.00)
2.	Amoxicillin (Am)	30	40 (78.43)	8 (15.69)	3 (5.88)
3.	Amoxicillin + Clavulanic acid (Ac)	20+10	35 (68.63)	6 (11.76)	10 (19.61)
4.	Azithromycin (At)	15	17 (33.33)	13 (25.49)	21 (41.18)
5.	Chloramphenicol (C)	30	24 (47.06)	6 (11.76)	21 (41.18)
6.	Ciprofloxacin (Cf)	5	0 (0.00)	5 (9.80)	46 (90.20)
7.	Ceftriaxone (Ci)	30	22 (43.14)	13 (25.49)	16 (31.37)
8.	Gentamicin (G)	10	0 (0.00)	2 (3.92)	49 (96.08)
9.	Kanamycin (K)	30	51 (100.00)	0 (0.00)	0 (0.00)
10.	Nalidixic acid (Na)	30	27(52.94)	17(33.33)	7(13.73)
11.	Norfloxacin (Nx)	10	10 (19.61)	18 (35.29)	23 (45.10)
12.	Nitrofurantoin (Nf)	300	45 (88.24)	1 (1.96)	5 (9.80)
13.	Ofloxacin (Of)	5	5 (9.80)	15 (29.42)	31 (60.78)
14.	Streptomycin (S)	10	0 (0.00)	0 (0.00)	51 (100.00)
15.	Tetracycline (T)	30	48 (94,12)	2 (3.92)	1 (1.96)
16.	Cotrimoxazole (Co)	1.25/23.75	32 (62.74)	8 (15.69)	11 (21.57)

Table 2. Drug resistance pattern among multi-drug resistant *Salmonella* isolates

R-group	Resistance Phenotype	No. of Isolates (%)
I.	A, C, Co	20 (39.21)
II.	A, C, T	24 (47.06)
III.	A, C, Na	11 (21.57)
IV.	A, C, Co, T	8 (15.69)
V.	A, C, Ci	17 (33.33)
VI.	A, C, Na, Ci	6 (11.76)

Figures in parenthesis indicate percentage (A)=Ampicillin, (C)=Chloramphenicol, (T)=Tetracycline, (Co)=Cotrimoxazole, (Na)=NalidixicAcid, (Ci)=Ceftriaxone.

Table 3. *In vitro* transfer of resistance pattern from donor to recipient

Resistant pattern of the donor	Isolates	Transfer Temperature (°C)	Resistance pattern transferred
ACCoT	177	37˚C	A C Co T
	186	37˚C	C T
	115	37˚C	A C Co T
	124	37˚C	A C Co T

(A)=Ampicillin, (C)=Chloramphenicol, (T)=Tetracycline, (Co)=Cotrimoxazole**.**

Table 4. MIC of selected antibiotics before and after conjugation studies from *Salmonella* spp. to *E. coli.*

Salmonella Isolates	Antibiotics	MIC before conjugation *Salmonella* Isolates (µg/ml)	MIC before conjugation *E. coli* (µg/ml)	MIC after conjugation *E. coli* (µg/ml)
	Ampicillin	500	S	500
	Chloramphenicol	120	S	60
S177	Cotrimoxazole	1000	S	1000
	Tetracycline	30	S	30
	Ampicillin	4000	S	S
	Chloramphenicol	120	S	120
S186	Cotrimoxazole	2000	S	S
	Tetracycline	120	S	120
	Ampicillin	2000	S	2000
	Chloramphenicol	500	S	500
S115	Cotrimoxazole	2000	S	2000
	Tetracycline	120	S	60
	Ampicillin	2000	S	2000
	Chloramphenicol	250	S	250
S124	Cotrimoxazole	1000	S	1000
	Tetracycline	120	S	120

S= sensitive

DISCUSSION

About 16 million cases of typhoid fever are reported each year 1.3 billion cases of gastroenteritis and 3million deaths occur worldwide due to *Salmonella* (Bhunia, 2008). Diagnosis of typhoid fever is done by isolating the causative bacteria from the patient most often from blood, but also from urine, stool, or bone marrow. Infected persons can develop sustained fever of up to 104°F (40˚C), weakness, stomach pain, and headache. A rash (rose spots) may accompany the infection (Huang *et al.,* 2009).In the present study 66.26% samples tested positive by Widal test while only 20.23% were culture positive. In contrast only 3.2% samples were found culture positive for *Salmonella* in the study of Peletiri and Ibecheozor (2012). A wide variation in the antibiotic susceptibility pattern was recorded in the study. Majority of the isolates were found resistant to Tetracycline (94.12%) and Ampicillin (92.16%) followed by Nitrofurantoin (88.24%), Amoxicillin (78.43%) and Amoxicillin and Clavulanic acid (68.63%) while 100% resistance was recorded for Kanamycin in the present investigation. Drug resistance in typhoid *Salmonella*e is considered as one of the important factors in the morbidity and mortality of the disease. In agreement with the study high levels of resistance among *Salmonella* isolates were recorded for Tetracycline (21.0-40.9%), Ampicillin (19.0% - 100.0%) and Amoxicillin previously (Akbarmehr 2012). In contrast none of the clinical isolates of *Salmonella* in the study of Cabrera *et al.,* (2004) demonstrated resistance to Amoxicillin Clavulanic acid. The isolates in the study showed sensitivity to Ciprofloxacin, Gentamycin and Streptomycin. Similar observations have been reported in other studies (Kabra *et al.,* 2000; Akbarmehr, 2012). However, varying degree of resistance to ciprofloxacin have been recorded in several researchers conducted elsewhere (Parry *et al.,* 2010 and Sule *et al.,* 2012). Since Nalidixic acid resistance is a marker for predicting low level resistance to Ciprofloxacin among *S. typhi* and also an indicator of treatment failure to Ciprofloxacin; correlation between resistance to Nalidixic acid and reduced susceptibility to ciprofloxacin and other Fluoroquinolones has been reported (Hakanen *et al.,* 1999). Therefore, as per the recommendation of CLSI (Wayne, 2006) all *S. typhi* isolates should be screened for Nalidixic acid resistance along with Ciprofloxacin. Any isolate showing resistance to Nalidixic acid should be reported as intermediately susceptible to Ciprofloxacin. Such strains have been found to be endemic in different parts of the world including India (Mandal *et al.,* 2004).

In India, *S. typhi* drug resistance has been reported since 1960, followed by the first outbreak of multidrug resistant *S. typhi* in Calicut (Panicker and Vimla, 1972). Since then MDR *S. typhi* has appeared throughout the world, especially in South America, the Indian sub-continent, Africa and south East Asia (Mourad *et al.,* 1993). This was in agreement with the present study where 39.21% isolates were multidrug resistant to Ampicillin, Chloramphenicol and Co-trimoxazole. Further as observed is the study, Mandal *et al.* (2002) also reported various resistance patterns in *S. typhi* isolates (ACT, ACNa, ACCoT, ACCi and ACNaCi). In the present study agarose gel electrophoretic analysis revealed the presence of plasmid of size corresponding to 600 bp and conferring ACCoT resistance in *S. typhi.* Several studies (Jesudasan *et al.,* 1996) from different parts of the world have reported plasmid mediated resistance of A, C, Co and T in *S. typhi* isolates. In addition to resistance to ACCoT, various, resistance patterns among MDR *Salmonella* isolates were observed in the present study. In the present study conjugation experiments demonstrated transfer of 600 bp plasmid encoded ACCo and T resistance determinants to *E. coli*. In recent years several authors have studied on multidrug resistance and transfer of *Salmonella* R-plasmids *via* conjugation. Hart *et al.* (1996) who studied non-typhoidal *Salmonella* resistant isolates demonstrated that 67.7% of the transferred their resistance to one or more antimicrobials to *E. coli* K12. Mandal *et al.* (2003) reported that in *S. enterica* serovar Typhi, the R-plasmid encoded resistance to Ampicillin, Chloramphenicol, Cotrimoxazole and Tetracycline was transferable. Several studies have demonstrated that R- plasmid transfer *via* conjugation between *Salmonella* isolates and other Gram negative bacteria has an important role in antimicrobial resistance of enteric bacteria (Poole *et al.,* 2009). Akbarmehr (2012) demonstrated that horizontal transfer of antibiotic resistance plasmids *via* conjugation can occur among *Salmonella* isolates with poultry origin. According to Mandal *et al.* (2004) resistance plasmid might have been transferred from other enteric bacteria in human, because human carries or patients are the source of *S. typhi* infection. Two factors have been identified to play role in acquisition of R-plasmids *i.e*., the capacity of the antibiotic resistance transposon to spread between plasmids and the selection exerted antibiotic treatment of enteric fever.

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