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Full Length Research Paper

Plasmid DNA analysis and conjugative study of antibiotic resistant *Salmonella typhi* and *paratyphi* to ten selected antibiotics in Zaria, Nigeria

Adeshina Gbonjubola Olusesan*, Osuagwu Nonyelum, Okeke Claire-Lorentz Ebere and Ehinmidu Joseph Olorunmola

Department of Pharmaceutics and Pharmaceutical Microbiology, Ahmadu Bello University, Zaria, Kaduna State, Nigeria.

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The antibiotic susceptibility of *Salmonella typhi* and *Salmonella paratyphi* isolates to ten selected antibiotics, plasmid DNA profiles and conjugative ability in Zaria, Nigeria were investigated. Blood samples collected from presumptive typhoid fever patients in the different locations were cultured for *Salmonella* species and identified by standard procedures. Susceptibility testing and minimum inhibitory concentration determination were performed using appropriate microbiological methods. Conjugative experiment was carried out with multiple antibiotics resistant isolates of *Salmonella* species. The resistant test bacteria strains were subjected to DNA isolation and characterization. Susceptibility study of test bacteria to ten selected antibiotics showed high percentage resistance to nine antibiotics such as Ampicillin, Amoxicillin, Augumentin, Chloramphenicol, Co -trimoxazole, gentamicin, Nitrofurantoin and Tetracycline (40 - 100%). Antibiotics resistance profile of *S. paratyphi* isolates were observed to be considerably higher than *S. typhi* isolates. The result of conjugation studies of multiple antibiotics resistant *Salmonella* species with Ofloxacin sensitive *Escherichia coli* ATCC 25722 showed that eight of the 18 Ofloxacin resistant *Salmonella* species isolates possess transferable resistant trait. The multiple antibiotics resistant (MAR) of test bacteria showed transferable plasmid sizes of 23.13 and 0.145 kb conferring resistance to the ten selected antibiotics. The findings from the plasmid analysis showed that the antibiotic sensitive *S. typhi* strains could acquire the R-plasmid from any resistant enteric bacteria such as *E. coli*, to undergo a suitable adaptation for survival in the changing antibiotic environment.

Key words: Plasmid, antibiotics, resistance, *Salmonella* species.

INTRODUCTION

It is estimated that each year there are approximately 21.6 million cases of typhoid fever, which result in 200,000 deaths worldwide (Curtis and Wheeler, 2006). In many developing countries in the tropical parts of the world, typhoid fever remains an important cause of morbidity and mortality, with an estimated annual global incidence of 21 million cases and more than 700,000 deaths. However, the incidence of cases and death has been greatly increased by a combination of poor sanitation and hygiene, unavailability of vaccines and high cost of effective antimicrobial chemo-therapy. Besides, the effectiveness of antimicrobial chemotherapy is also being challenged by the emergence of antibiotic resistance (Woodford and Ellington, 2007; Clewell, 2008). In Nigeria, the antibiotics most readily available for treatment of typhoid are Chloramphenicol, Ampicillin and Co-trimoxazole. With the emergence of plasmid-encoded Chloramphenicol resistance (Mandal et al., 2004), Ampicillin, although slightly less effective than Chloramphenicol, was used both for therapy and for elimination of the carrier state (Cooke and Wain, 2004). Again, plasmid- encoded resistance soon developed (Mandal et al., 2004) . Finally, Co-trimoxazole was introduced in 1980 and plasmid-encoded resistance to Trimethoprim and Sulfonamides was observed shortly afterwards (Aaerestrup, 2006).

*Corresponding author. E-mail: dotunkele@yahoo.com. Tel: 080 37880000.

The first cases of typhoid due to *Salmonella enterica* carrying plasmid-encoded resistance to Chloramphenicol, Ampicillin and Co-trimoxazole were reported from East and South Asia (Mirza et al., 2000). There is also a potential epidemic zone in the Middle East (Kuwait, Oman and Saudi Arabia) due to importation of multiple antibiotics resistant *S. enterica* by migrant workers from the epidemic zone (Mirza et al., 2000). Interestingly multi-antibiotics resistant *S. enterica* is rarely, reported from Africa (South and Central) (Mirza et al., 2000). In relation to effective surveillance and the development of rational control stra-tegies for this important human disease, the availability of detailed and accurate data related to the molecular epidemiology of *Salmonella* species is crucial. Typhoid and paratyphoid fever remain an important, but under estimated disease which has high mortality in developing countries. However, there are extremely limited data from Zaria, Nigeria, hence the probable under -estimation of the disease burden in the area. Therefore, there is need to investigate the antimicrobial susceptibility and the incidences of plasmid mediated resistance of multi-antibiotics resistant *Salmonella* species isolates from Zaria, Nigeria.

MATERIALS AND METHODS

Bacterial strains

The *S. typhi* and *S. paratyphi* A and B were isolates from blood sample of patients who were presumptively diagnosed for typhoid fever from Ahmadu Bello University Clinic, Federal College of Education Clinic, Salama Hospital and Savanna Polyclinic Zaria, Nigeria were used as test *Salmonella species* isolates. Each isolates were characterized to species level; *S. typhi* or *S. paratyphi* as described by Willey et al. (2008).

Susceptibility tests

The susceptibility of Salmonella species isolates to selected commonly prescribed antibiotics was performed using Kirby Bauer disc diffusion method (Cheesbrough, 2000). Overnight cultures of *Salmonella* species in nutrient broth were standardized to 1.0 x 10^6 cells/ml and flooded over the prepared Mueller- Hinton agar plates. Excess were drained off and allowed to dry in a warm incubator for about 15 - 20 min. Antibiotics impregnated disc of known con-centrations; Ampicillin (20 g), Amoxicillin (25 g), Augumentin (30 g), Chloramphenicol (30 g), Co-trimoxazole (25 g), Gentamicin (10 g), Nalidixic acid (30 g), Nitrofurantion (300 g), Ofloxacin (30 g) and Tetracycline (30 g) were applied on the inoculated agar plates aseptically. Plates were incubated at 37°C for 24 h. Interpretation of strains as sensitive or resistance were based on zones of inhibition according to NCCLS standards (BSAC, 2002; NCCLS, 2002) in accordance with WHO requirement.

Determination of minimum inhibitory concentration (M.I.C.)

The minimum inhibitory concentration was determined by the agar dilution method. Graded concentrations of the test antibiotics (Ofloxacin, Amoxacillin, Gentamicin and Chloramphenicol (0.25 - 1280 g/ml) were prepared and mixed aseptically with freshly prepared double strength Muller-Hinton agar in plates. The over-night cultures of test *Salmonella* species were standardized to 1.0 x 10^6 cells/ml. The test antibiotic-Muller Hinton Agar admixtures in plates were inoculated with 20 µl of standardized overnight culture of test *Salmonella species* isolates. The plates were incubated at 37°C for 24 h. The lowest concentration of test antibiotics that showed no growth was taken as the MIC of that antibiotic. The peak plasma level of 5 g/ml amoxicillin, 3 - 4 g/ml for gentamicin, ofloxacin > 4 /ml and 10 g/ml of chloramphenicol were used to determine the break point according to Sweetman (2005).

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), with slight modification described elsewhere (Mandal et al., 2003). Ofloxacin was used as selection agent.

The minimum inhibitory concentractions (M.I.C.) of the test antibiotics against the sensitive *E. coli* were determined. The resistant isolates of *S. typhi* were grown in sterile nutrient broth (5 ml) each at 37°C for 18 h. The ofloxacin sensitive *E. coli* were sub-cultured into the sterile nutrient broth and incubated at 37°C for 18 h. The overnight cultures of the potential donor (R^+) *S. typhi* and *S. paratyphi* resistant isolates and the competent recipient (R^-) that is, sensitive *E. coli* were grown in a ratio 10: 1 respectively in 50 ml volume of sterile nutrient broth and incubated in a static incubator at 37°C for 18 h. The loopful of trans-conjugant from the test organisms isolates admixtures (*S. typhi, S. paratyphi* and *E. coli*) bottles were sub- cultured in triplicates on MacConkey agar plates incorporated with the antibiotics M.I.C. (µg/ml) against sensitive *E. coli* and incubated at 37°C for 24 h. The plates were examined for the presence or absence of cultural characteristics of *E. coli* and lactose fermenting properties. The colonies of *E. coli* observed were aseptically picked and transferred into nutrient agar slant and sub-cultured. After which the M.I.C. of trans-conjugants were determined as described by Lennette et al. (1990).

Isolation of plasmid DNA and agarose gel electrophoresis

The multiple antibiotics resistant (MAR) isolates of *Salmonella species* and the antibiotic sensitive *Salmonella species* isolates were subjected to plasmid DNA isolation according to the protocol of Birnboim and Doly (1979); Kado and Liu (1981) with some modifications. Agarose gel electrophoresis of the isolated plasmid DNA was carried out in tris-borate buffer system, using 1.2% agarose, for 1 h at 75v.

(a) The plasmids DNA were isolated using lysing solution. The lysate were kept in ice for 30 min and centrifuged for 5 min, phenol: chloroform (1:1) treatment was followed with the clear supernatant. Plasmid DNA were precipitated with equal volume of chilled isopropyl alcohol and DNA pellet dissolved in 100 µl of TE buffer (diethylether).

(b) A 0.8% agarose gel was used to resolve DNA fragment and it was prepared by combining 0.8 g agarose in ten times concentration of Tris acetate ethylene diamine tetraacetate (10 ml 10XTAE) buffer and 90 ml distilled water in a 250 ml beaker flask and heating in a microwave for 2 min until the agarose is dissolved. 2.5 ml ethidium bromide (5.0 mg/ml) was added to the dissolved agarose solution with swirling to mix. The gel was then poured onto a mini horizontal gel electrophoresis tank and the casting combs were inserted. It was then allowed to gel for 30 min. The casting comb was then carefully removed after the gel had completely

Table 1. Percentage zones of Inhibition by antibiotics against Isolated *Salmonella* species.

Test	Antibacterials	Resistance n (%)	
	Antibiotics	***S. typhi* (n=71)**	***S. paratyphi* (n=31)**
1	Amoxicilin	63(88.7)	28(90.32)
2	Ampicilin	64(90.1)	29(93.54)
3	Augumentin	62(87.3)	31(100)
4	Chloramphenicol	43(60.6)	17(54.84)
5	Cotrimoxazole	40(56.3)	16(51.61)
6	Gentamicin	42(59.2)	22(70.97)
7	Nalixidic acid	29(40.9)	15(48.39)
8	Nitrofurantoin	39(54.9)	19(61.29)
9	Ofloxacin	7(9.9)	10(32.26)
10	Tetracycline	56(78.9)	27(87.10)

Table 2. Susceptibility of test *Salmonella species* isolates (10^6 cfu/ml) to four selected antibiotics, using test antibiotic peak plasma level.

Test antibacterials	**Resistance n (%)**		
Antibiotics	***S. typhi* (n = 71)**	***S. paratyphi* (n = 31)**	**Significant level**
Amoxicilin	61(86)	28 (90.32)	$p < 0.05$
Chloramphenicol	39 (55)	21 (67.74)	$P < 0.05$
Gentamicin	40 (56)	23 (74.19)	$P < 0.05$
Ofloxacin	11 (15)	14(45.16)	$P < 0.05$

solidified. One times concentration (IX) TAE electrophoresis buffer was then added to the reservoir until the buffer just covered the agarose gel.

0.5 µl of gel tracking dye (bromophenol blue) was added to 20 µl of each sample with gentle mixing. 20 µl of the sample was then loaded onto the wells of the gel, the mini horizontal electrophoresis gel set-up was then covered and the electrodes connected. Electrophoresis was carried out at 100 - 120 mA for 1 h. At the completion of the electrophoresis, the gel was removed from the buffer and gel was viewed under a long wave UV-light box. The band pattern of the DNA fragments were then photographed with a Polaroid camera and documented using an electrophoresis gel documentation system. The molecular sizes of each plasmid were determined by comparison with plasmids of known mass (Datta et al., 1971).

RESULTS

The antibiotics susceptibility profile of one hundred and two strains of *Salmonella species* (*S. typhi* and *S. paratyphi*) isolated from typhoid suspected patients as showed in Table 1, clearly illustrate the marked difference in sensitivity profile of test bacteria. *S. paratyphi* showed higher resistance to the selected antibiotics than *S. typhi.* The highest resistances observed are the multiple antibiotic resistances to the beta-lactam antibiotics.

Percentage resistance ranged from 60 - 90% among *S. typhi* and *S. paratyphi*. *S. typhi* and *S. paratyphi* recorded 100% resistance to Augumentin. *Salmonella species* was also observed to be resistant to the activity of the commonly prescribed antibiotic in urinary tract infection (Nitrofurantoin). A very large number of the *Salmonella species* were observed to exhibit resistance to tetracycline. Chloramphenicol a first line drug in the therapy of typhoid fever infection was remarkably less effective. Though less active, it is relatively more effective than the -lactam antibiotics investigated in this study. Generally, the *S. paratyphi* exhibited higher resistant profile to the studied antibiotics.

Ofloxacin widely known for its effectiveness in typhoid fever chemotherapy registered 15% resistance against *S. typhi* isolates (Table 2) with as high as 45% resistance to *S. paratyphi*.

Note

Test antibiotic M.I.C. break points against Gram negative bacteria isolates using its peak plasma values according to British pharmacopiea; Amoxicillin > 5 g/ml, Chloramphenicol > 10 g/ml, Gentamicin > 4 g/ml,

Table 3. Conjugation studies using Ofloxacin on Ofloxacin sensitive *E.coli* ATCC 25722 (10^6 cfu/ml)

	Isolates	MIC before conjugation (mg/ml)	*E.coli* MIC before conjugation (mg/ml)	*E. coli* characteristics	MIC after conjugation (mg/ml)
1	SF20	64	1.0	+	64
2	210	8	1.0	+	8
3	211	256	1.0	+	256
4	277	32	1.0	-	-
5	341	64	1.0	-	-
6	342	512	1.0	+	512
7	373	32	1.0	-	-
8	391	128	1.0	-	-
9	399	8	1.0	-	-
10	403	128	1.0	-	-
11	407	16	1.0	-	-
12	408	16	1.0	+	16
13	422	128	1.0	+	64
14	433	256	1.0	+	256
15	435	32	1.0	-	-
16	469	4	1.0	+	4
17	473	16	1.0	-	-
18	602	8	1.0	-	-

M.I.C range 0.5 - 512 g/ml.

Table 4. *Salmonella species* isolates, antibiogram profiles and transfer of resistant determinants into *E. coli* ATCC 279522.

Bacterial isolates lab no.	Conjugants MIC(g/ml)	Transfer temperature(°C)	Transfer resistant determinants
SF20	64	37	Am,Ap,Au,Cm.Co,Ge, Ni,Nx,Of,Tc
210	8	37	Am,Ap,Au,Cm,Ge,Nx,Tc
211	256	37	Am,Ap,Au,Cm,Co,Ge,Nx,Of,Tc
342	256	37	Am,Ap,Au,Cm,Co,Ni,Nx,
408	16	37	Am,Ap,Au,Cm,Co,Ge,Ni,Tc
422	64	37	Am,Ap,Au,.Cm,Ge,Nx,Of,Tc
433	256	37	Am,Ap,Au,Cm.Co,Ge, Ni,Nx,Of,Tc
469	4	37	Am,Ap,Au,Cm.Co,Tc

Key: Am, Amoxicilin; Ap, Ampicillin; Au, Augumentin; Cm, Chloramphenicol; Co, Cotrimoxazole; Ge, Gentamicin; Ni, Nitrofurantion; Nx, Nalidixic acid; Of, Ofloxacin; Tc, Tetracycline.

Ofloxacin > 4 g/ml.

The result of the conjugation studies carried out to determine the existence of transferable multiple antibiotics resistant determinant using Ofloxacin resistant *S. typhi* isolates as the selection agent (Table 3) shows that out of the eighteen (18) isolates with Ofloxacin resistant factor, only eight showed ability to transfer its resistant factor as was determined through the increase in the minimum inhibitory concentration of the recipient *E. coli* strain.

The anti-biogram before and after conjugation showed the same phenotypic resistant pattern (Table 4).

Curing of the transconjugants with acridine orange showed changes in the antibiotics resistant pattern of all tested transconjugants. The minimum inhibitory concentration values of the tested transconjugants decreased significantly when compared with those obtained in untreated transconjugants (Table 5).

Only four of the *Salmonella species* isolates were found to be harboring plasmids and the estimated molecular sizes of the plasmid were 23.13 and 0.145 kb (Table 6).

The plasmid DNA isolated from the transconjugants co-migrated with the plasmid isolated from their corresponding donor strains and is about 23.13 kb. The antibiotic sensitive strains of *S. typhi* did not show any plasmid band in the gel (Figure 1).

Table 5. Curring *E.coli* transconjugants.

	Isolates lab no.	MIC before curring transconjugants(g/ml)	MIC after curring transconjugants(g/ml)
1	SF20	64	1.0
2	210	8	1.0
3	211	256	1.0
4	342	256	1.0
5	408	16	0.5
6	422	64	1.0
7	433	256	1.0
8	469	4	1.0

Table 6. Plasmid profiles of the *Salmonella* species isolates harbouring plasmids.

Salmonella species isolate lab no.	Plasmids number observed	Estimated molecular sizes of plasmid (kb) observed
277	2	23.13, 0.145
342	2	23.13, 0.145
300	2	23.13, 0.145
399	2	23.13, 0.145

DISCUSSION

Salmonella species isolates obtained from blood samples of suspected typhoid patients in the student and private clinics showing multiple antibiotic resistant were conjugated to determine whether their resistant determinant was resident on plasmid or it was chromosomal. The transconjugant were selected using Ofloxacin. Interestingly, on antibiotic susceptibility testing, the transconjugants exhibited the same resistant pattern before conjugation in addition to Ofloxacin resistance. The minimum inhibitory concentrations of the transconjugants were similar to that of their corresponding donor strains. As observed from the result, it is likely that selective pressure on the intestine commensal bacteria due to inappropriate diagnosis and prescription of antibiotics might be the source of dissemination of the plasmid conferring resistance to the ten (10) selected antibiotics in *Salmonella species* therapy. This is because most of these isolates from private and student clinics showed a common resistant pattern and coupled with the fact that their resistant factors were transferable. Furthermore, the resistance isolates and their corresponding *E.coli* transconjugants co-migrated in electrophoresis tank having the same band pattern.

Agarose gel electrophoretic analysis revealed the presence of plasmid of size approximately the same among the isolates associated with enteric fever in private and student clinics in Zaria, Nigeria. The corresponding transconjugants also showed the same plasmid size on agarose gel electrophoresis. Agarose gel electrophoretic analysis revealed the presence of plasmids of approximately 23.13 and 0.145 kb among the isolates associated with multiple antibiotics resistant strains. The corresponding transconjugants also contained the similar plasmid. The same plasmid patterns of multiple antibiotics resistance isolates from the student and private clinics suggest the wide spread occurrence of plasmid mediating antibiotic-resistance. This phenomenon indicated the existence of a plasmid carrying multiple antibiotics -resistances in the bacterial population in Zaria, Nigeria. In the studies, there was co-existence of antibiotic-sensitive and multiple antibiotics resistance strains of *Salmonella* species, but unlike multi-ple antibiotics resistance strains, the sensitive strains did not contain any plasmid. The presence of conjugative R-plasmid in multiple antibiotics resistant *Salmonella* species and absence of any plasmid in the sensitive strain has been reported earlier by researchers from Kolkata, India (Mandal et al., 2004; Shyamapada et al., 2006). Thus, in *Salmonella* species the R-plasmid is an unstable plasmid that may appear or disappear at any time resulting in the emergence of drug resistant or drug sensitive isolates. The selection exerted by indiscriminate use and sub-standard antibiotics treatment of enteric fever may be the cause of acquisition of R-plasmid (Mandal et al., 2003). Through the acquisition of a plasmid conferring multi-drug resistance, the strain undergoes the necessary and appropriate adaptation for survival in the changing antibiotic environment. Thus, it appears that the already existing sensitive strain, by the acquisition of an R-plasmid, has emerged as a resistant strain within the *Salmonella species* bacterial population

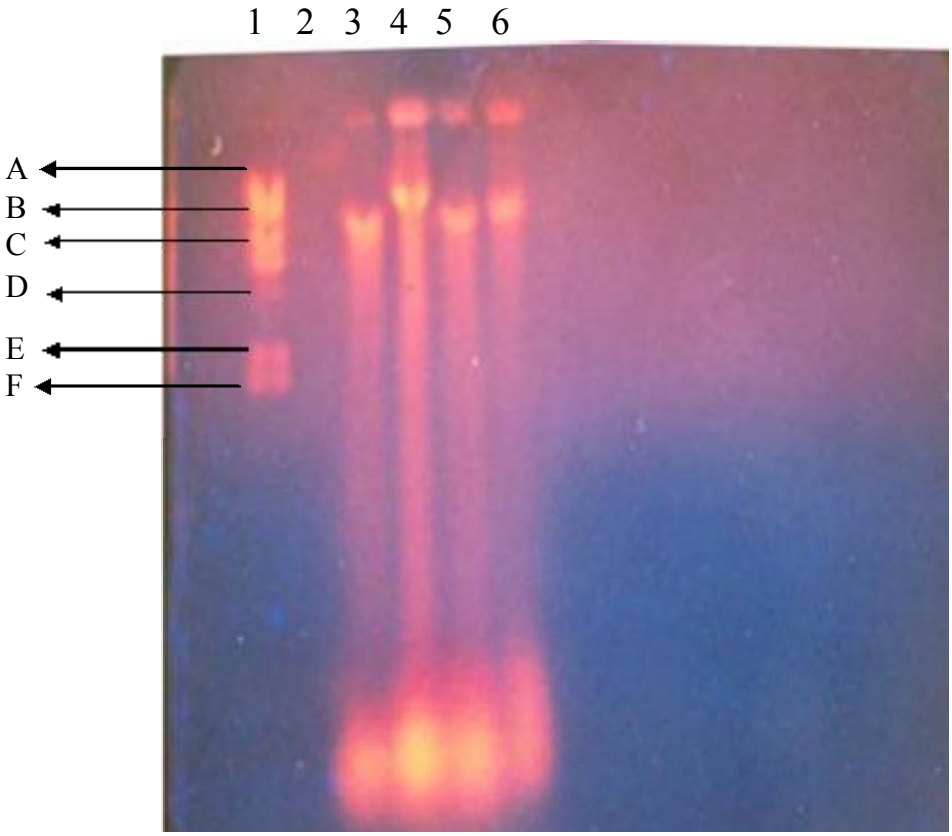

Figure 1. 1.2% Agarose gel electrophoresis of plasmid DNA isolates from multiple antibiotic resistance *Salmonella species* in Zaria.
Lane 1: Molecular weight standard Hind marker showing plasmid sizes. A: 23.13 kbp, B: 9.416 kbp, C: 6.5576 kbp, D: 4.361 kbp, E: 2.322 kbp, F: 2.09 kbp. Lane 3: *Salmonella typhi* isolate Lab No 277. Lane 4: *Salmonella typhi* isolate Lab number 342. Lane 5: *S. paratyphi* isolate Lab number 300. Lane 6: *S. typhi* isolate number 399.

in and around Zaria, Nigeria has been able to adapt the challenge of antibiotics. The prevalence of *S. typhi* harbouring the plasmid encoding antibiotics resistance in Zaria, Nigeria has not yet been widely reported. From the results of the *in vitro* conjugation experiments, there is the possibility of acquisition of R -factor from intestinal flora like *E. coli. In vitro* and *in vivo* acquisitions of R -plasmids from common intestinal bacterial flora by *S. typhi* have been reported earlier (Mandal et al., 2003). Rubin, (2001) also reported the acquisition of R- plasmids by *S. typhi* accounting for hepatic enlargement in patient with enteric fever. The multiple antibiotics resistant isolates with similar antibiogram isolated from different locations within Zaria, Nigeria are of great importance. Thus, the phenomenon indicates the existence of transferable resistance plasmid pool in the bacteria population in Zaria, Nigeria. It has been reported that the occurrence of plasmid bearing one or more resistance gene such as the R-plasmid observed in this study possesses the ability to often code for enzymes that destroy or modify drugs; example is the hydrolysis of penicillin or the acetylation of Chloramphenicol and Aminoglycosides drugs (Miguel, 2004). The high resistance of *Salmonella species* isolates may be due to the presence of R-plasmid coding for more than one resistant gene. According to Willey et al. (2008), once a bacterial cell possesses an R- plasmid, the plasmid may be transferred to other cells quite rapidly through normal gene exchange processes such as conjugation, transduction and transformation. This gene exchange may have led to a single plasmid carrying gene for resistance to several antibiotics.

Conclusion

In conclusion, multiple antibiotics resistant *Salmonella species* isolates were observed to be prevalent. Plasmid analysis showed that the antibiotic sensitive *S. typhi* isolates could acquired the R -plasmid from other enteric bacteria such as *E.coli* for a suitable adaptation for survival in the changing antibiotic environment. This observation could result in high level of therapeutics failure in typhoid patients in Zaria, Nigeria.

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