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Full Length Research Paper

Infectious drug resistance plasmid study in *Salmonella enterica* isolates of domestic animals and lizards from some south western states of Nigeria

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Twenty-three *Salmonella enterica* isolated from domestic animals and lizards in Oyo and Ogun States of Nigeria between April 2005 and August 2007 were studied for infectious drug resistance and possible transfer using *Escherichia coli* 365K12 resistant to 200 µg/ml streptomycin as sensitive recipient. Nineteen (79%) of the isolates were from poultry, 2 (8%) from cattle and 2 (8%) from lizards. Three of the poultry isolates 3/19 (15.8%) transferred R-factor for tetracycline, 10/19 (52.6%) for ampicillin,6/19 (31.6%) for kanamycin, 10/19 (52.6%) for neomycin and 2/19 (10.5%) for nalidixic acid, two of the cattle isolates, 2/2 (100%) transferred for tetracycline, ampicillin, kanamycin, neomycin, ½ (50%) for chloramphenicol and ciprofloxacin. Two of the isolates, 2/2 (100%) from lizard transferred for tetracycline, ampicillin, kanamycin, and neomycin; 1/2 (50%) for chloramphenicol and nalidixic acid. The occurrence of a high level of infectious drug resistance among *Salmonella enterica* studied that were transferable to sensitive recipient *E. coli* 365 K12 is of public health concern in terms of possible transfer of drug resistance from animal to human.

Key words: *Salmonella enterica,* infectious drug resistance, plasmid transfer, antimicrobials.

INTRODUCTION

The abuse and overuse of antimicrobials in both human and veterinary medicine have been associated with the spread of multiple antimicrobial resistances. This phenolmenon has been reported worldwide in *Salmonella enterica* (SE) an organism implicated in severe enteritis in humans (Carattoli, 2003).

The dissemination of multiple drug resistance has been largely attributed to conjugative DNA exchange which occurs between plasmids and the bacterial chromosome (Burland et al., 1998; Waters, 1999). The integration of resistance genes into specialized genetic elements, called integrons, usually play a major role in acquisition and dissemination of resistance genes (Carattoli 2003), but when resistance genes are located on plasmids, they can be mobilized by conjugative transfer (Waters, 1999). Resistance genes are often located on extra chromosomal genetic elements or in segments inserted within the chromosome that originates from other genomes (Waters, 1999). The acquisition of a new gene may occur by genetic transformation.

S. enterica isolates have been characterized by the presence of host-adapted virulence plasmids encoding genes contributing to colonization and resistance to complement killing, such as the spvA, spvB and spvC (salmonella plasmid virulence) and the RCK (resistance to complement killing) genes (Guiney et al., 1994). It has been observed that *Salmonella* obtained from both food animals and humans shows increasing antimicrobial resistance rates (Hakanen et al., 2005). For instance, *S. enterica* strains with multiple antibiotic resistances (to four or more antimicrobials) are now widespread in both developed and developing countries, of which most of the strains are zoonotic in origin (Threlfall, 2002).

Majority of multiple antimicrobial resistance *S. enterica* strains acquired their resistance in the food-animal host and they also caused human infections through the food

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Abbreviations: CHL, Chloramphenicol; **STR,** streptomycin; **TET,** tetracycline; **MIC,** minimum inhibitory concentration.

chain (Threlfall, 2002). The possibility that plasmid transfer can occur inside animals has been considered since the 1960s, usually as a potential scenario for antibiotic resistance dissemination (Kasuya. 1964). Salmonella infections have been associated with the ingestion of poultry, meat, milk and dairy products (Bean et al., 1996).

In Nigeria, Adetosoye (1980) reported that while, *Salmonella glostrup* transferred CHL and STR resistance determinant, *Salmonella avonmouth* strains transferred STR resistance determinant, to sensitive *Escherichia coli* K12 recipients. He further observed that strains of *Salmonella labadi* (from fowls), *Salmonella Dublin* (from cow), *Salmonella tokoradi* (from Australian skunk), *Salmonella aba* (from elephant), *Salmonella virchow* (from cow), *Salmonella typhimuriium* from gorilla, (one from green monkey) and *Salmonella agama* (from dog) did not transfer their R determinants to sensitive *E. coli* K12 recipients. However two strains of *Salmonella saint paul* one from pig and one from fowl transferred TET, STR and TET R determinant respectively. Also one strain of *S patience* (from dog) transferred TET, SUL and two strains of *S. labadi* (one from pig and one from fowl) transferred TET and SUL, STR R determinant respectively to the sensitive *E coli* K12 recipients.This work investigated the occurrence of *S. enterica* isolates from poultry, cattle and lizards, R plasmid harbored by *S. enterica* isolates and lizard transferable to sensitive recipient *E. coli* K12. Implication of the findings is discussed.

MATERIALS AND METHODS

Salmonella isolates

A total of 800 animals comprising of 300 and 100 poultry from Ogun State and Oyo State respectively, 200 and 100 cattle from Ogun State and Oyo State, respectively, while 100 lizards were sampled from Oyo State. The bird samples were from commercial layers and broilers presented for post mortem examination at University of Agriculture, Abeokuta and Department of veterinary pathology, post mortem complex, University of Ibadan, They died as a result of clinical signs of septicaemia characterized by depression, prostration, anorexia, high fever and death. At postmortem, petechial haemorrages and congestion of the affected organs was observed. Samples were aseptically collected from livers, kidneys, spleen, heart blood and bone marrow from the birds. The non diarrhoeic cattle were restrained in the metal cage and with the aid of sterile swabs fecal samples were collected aseptically. The lizards captured in poultry houses at the teaching and research farm, University of Ibadan, were captured with metal cage and restrained chemically by placing cotton wool containing diethyl-ether on the nostrils. All the samples were transported on ice (Coleman® Flask) to the research laboratory in the Department of veterinary microbiology and parasitology, University of Ibadan for analysis. The samples were analysed bacteriologically for *S. enterica* according to standard procedures as described by Barrow and Feltham (1993).

A total of 49 *S. enterica* isolates were recovered from the animals. However, 23 multidrug resistant *S. enterica* isolates sensitive to streptomycin were used for the infectious drug resistance study. The isolates were studied for antimicrobial susceptibility to, ciprofloxacin > 98% HPLC (17850-5G), kanamycin sulfate 785 µg/mg (N6386-5G), chloramphenicol (C1919-5G), streptomycin sulfate salt 775 units/mg (S6501-5G), neomycin trisulfate salt 689 µg/mg (N6386-5G), nalidixic acid sodium salt (N4382-5G), ampicillin sodium salt 91.5% - 100.5% (A9518-5G) and tetracycline hydrochloride (T7660-5G) (Sigma Aldrich, Inc., 3050 Spruce Street, St Loius, Mo63103 USA). All the antibiotic powders were supplied courtesy John Wain of Tropical Microbiology, Welcome Trust Sanger Institute Cambridge UK.

Determination of drug resistance (minimum inhibitory concentration)

The minimum inhibitory concentration (MIC) of Ciprofloxacin, Nalidixic acid, Ampicillin, Streptomycin, Chloramphenicol, Kanamycin, Neomycin, Tetracycline for each of the 49, *SE* isolates was determined respectively by microtitre method as previously described (Adetosoye and Rotilu, 1987). Known weight of each antibiotic powder mentioned above except Nalidixic acid and Chloramphenicol was dissolved in sterile distilled water to a final concentration of 70µg/ml, 64 µg/ml, 64 µg/ml, 64 µg/ml, 70 µg/ml and 60 µg/ml, respectively. Nalidixic acid was , however, dissolved with 2 drops of 0.2 M NaOH and made up to a final concentration of 64 µg/ml, with sterile distilled water, while Chloramphenicol powder was dissolved in 1 ml of 96% alcohol and made up to a final concentration of 200 µg/ml with sterile distilled water. All other procedures were as described by Adetosoye and Rotilu (1987). The procedure was repeated for all the tested antibiotics for the 49 *SE* studied. The MIC of the respective antibiotic was taken as the lowest concentration of the antibiotic that inhibits the growth of the *SE* isolate. The change in color indicated growth of the resistant isolate as seen in positive control and no color change was seen in sensitive strains. The well nearest to where there was color change (red formesan) was taken as the minimum inhibitory concentration of the antibiotic tested.

Transfer of resistance plasmids

Donor strains

Each of the 23 *S. enterica* isolates that showed resistance to any of the combinations of ciprofloxacin, nalidixic acid, ampicillin, chloramphenicol, kanamycin, neomycin, tetracycline but sensitive to streptomycin were used in the resistance transfer studies.

Recipient

E. coli 356 K12 resistant to 200 µg/ml of streptomycin courtesy Dr J.R. Walton, University of Liverpool was used as recipient.

Conjugation procedure

Twenty three *S. enterica* that were multidrug resistant but sensitive to streptomycin at MIC ≤ 16 µg/ml were used respectively as donors in the resistant plasmid transfer studies. The method used was that of Walton (1972) and modified by Adetosoye (1980).

A discrete colony of each resistant *SE* as well as *E. coli* 356 K12 was respectively inoculated into 4 ml of nutrient broth (Difco Laboratories, Detroit MI, 48232-7058, USA), contained in sterile test tubes with cork. The cultures were incubated aerobically in HEARSON® incubator (HEARSON Laboratory Equipment, HEARSON, Willow Walk, London S.E. 1) at 37°C for 10h. Subsequently 0.02 ml of the donor culture was delivered into sterile 10 ml nutrient broth (Difco Laboratories, Detroit MI, 48232-7058, USA) and 0.04ml of

Table 1. Minimum inhibitory concentration of *S. enterica* (SE) isolated from poultry, cattle and lizard sources against commonly used antimicrobials.

SE isolate code	Source of isolate	Antimicrobial/Minimum Inhibitory Conc. (ug/ml)							
		Cip	Chlo	Kan	Nal	Neo	Amp	Strep	Tet
A4	Poultry	1.88	0.78	7.5	100	35	35	1	0.55
A5	Poultry	1.88	0.78	7.5	1.56	>35	4.38	2	0.27
A6	Poultry	1.88	0.78	1.88	100	35	35	8	0.27
A7	Poultry	0.94	0.78	3.75	100	>35	4.38	16	0.27
B10	Cattle	0.47	1.56	>30	1.56	35	>35	1	>35
B15	Cattle	>30	100	30	25	>35	>35	16	>35
A24	Poultry	1.88	0.78	7.5	100	35	>35	16	35
A28	Poultry	0.23	100	30	0.78	>35	>35	8	>35
A29	Poultry	0.23	0.78	3.75	0.78	>35	8.75	4	2.19
A30	Poultry	0.23	0.78	1.88	0.78	>35	8.75	4	0.55
A31	Poultry	7.5	0.78	7.5	100	35	8.75	8	0.55
A32	Poultry	0.23	0.78	7.5	6.25	>35	8.75	1	1.1
A33	Poultry	0.23	0.78	30	3.13	>35	>35	1	0.55
A34	Poultry	0.47	0.78	30	3.13	35	>35	0.25	0.55
A35	Poultry	0.47	0.78	3.75	25	>35	8.75	0.5	0.55
A36	Poultry	1.88	0.78	30	3.13	35	17.5	0.5	0.55
A37	Poultry	7.5	6.25	15	3.13	>35	8.75	2	0.55
A38	Poultry	7.5	0.78	3.75	0.78	>35	8.75	1	0.55
C40	Lizard	>30	3.13	>30	6.25	>35	>35	2	>35
C41	Lizard	>30	50	>30	100	>35	>35	2	>35
A43	Poultry	0.47	0.78	7.5	6.25	>35	8.75	8	8.75
A44	Poultry	0.47	0.78	7.5	12.5	35	8.75	2	0.55
A45	Poultry	0.47	0.78	7.5	6.25	>35	8.75	8	1.1

Cip= Ciprofloxacin; Nal= nalidicic acid; Amp= ampicillin; Str= streptomycin; Chl= chloramphenicol; Kan= kanamycin; Neo= neomycin, and Tet= tetracycline.

of the recipient *E. coli* K12 was added. The mixture was similarly incubated aerobically at 37°C for 18h. The transconjugants were inoculated onto sterile MacConkey agar (Oxoid CM 109®) selective media with the formulation shown in (Table 4). The plates were incubated at 37°C overnight. All the transconjugants which grew on selective media were considered as having acquired resistance from the donor strains.

Selective media

The selective media were prepared by addition of 100 µg/ml of streptomycin and 50 µg/ml each of the antibiotics studied namely, ciprofloxacin, nalidixic acid, ampicillin, chloramphenicol, kanamycin, neomycin and tetracycline into 25 ml of MacConkey agar.

Antibiotic sensitivity of the transconjugant

The *in-vitro* antibiotic sensitivity test of each of the transconjugants was carried out as described by Walton (1972) and modified by Adetosoye (1984). A colony each of the transconjugants was inoculated into 5ml sterile nutrient broth and incubated at 37°C for 8 h.

The 0.01 ml portion of the culture was delivered into 4 ml of sterile nutrient broth and the mixture was vigorously shaken to give a 1:2000 dilution. Subsequently a diagnostic sensitivity test agar plate was inoculated by flooding with the 1:2000 diluted broths. The excess broth was drained off and the plate was allowed to stand on the bench for 1h after which antibiotic disc of ciprofloxacin (60 µg), chloramphenicol (200 µg), kanamycin (60 µg), nalidixic acid (20 µg), neomycin (60 µg), ampicillin (60 µg) and tetracycline (60 µg) were aseptically and respectively applied. The test plates were allowed to stand on the bench for 1 h to allow the antimicrobial agents to diffuse into the agar. The plates were then incubated at 37°C for 18 h after which the results were recorded.

RESULTS AND DISCUSSION

Forty-nine of the 800 (6.1%) animals examined for the presence of *S. enterica* isolates were positive. However, 23 of the 49 (46.9%) *S. enterica* isolates recovered from the studied animals were found to be multidrug resistant *S. enterica* isolates sensitive to streptomycin. Nineteen of the isolates, 19/23 (82.6%) were from poultry, 2/23 (8.7%) from cattle and 2/23 (8.7%) from lizard. The results of the minimum inhibitory concentration, antibiotic resistance patterns and antibiotic resistance transfer mode by trans-conjugants of the *S. enterica* isolates from animal sources are shown in Tables 1, 2 and 3, respectively.

Two poultry *S. enterica* isolates showed double R type-amp.neo, 1 had double R type-nal.neo, 1 showed triple R

Table 2. Antibiotic resistance patterns of the *S. enterica* isolates from animal sources to some commonly used antimicrobials.

SE isolate code	Source of the isolate	Antibiotic resistant pattern
A4	Poultry	Amp.Kan.Nal.Neo.Cip
A5	Poultry	Kan.Neo.Cip
A6	Poultry	Amp.Nal.Neo.Cip
A7	Poultry	Nal.Neo
B10	Cattle	Tet.Amp.Kan.Neo
B15	Cattle	Tet.Amp.Chl.Kan.Neo.Cip
A24	Poultry	Tet.Amp.Kan.Nal.Neo.Cip
A28	Poultry	Tet.Amp.Chl.Kan.Neo
A29	Poultry	Amp.Neo
A30	Poultry	Amp.Neo
A31	Poultry	Amp.Kan.Nal.Neo.Cip
A32	Poultry	Amp.Kan.Neo
A33	Poultry	Amp.Kan.Neo
A34	Poultry	Amp.Kan.Neo
A35	Poultry	Amp.Neo
A36	Poultry	Amp.Kan.Neo.Cip
A37	Poultry	Amp.Kan.Neo.Cip
A38	Poultry	Amp.Neo.Cip
C40	Lizard	Tet.Amp.Kan.Neo.Cip
C41	Lizard	Tet.Amp.Chl.Kan.Nal.Neo.Cip
A43	Poultry	Tet.Kan.Neo.Amp
A44	Poultry	Amp.Kan.Neo
A45	Poultry	Amp.kan.neo

Cip= Ciprofloxacin; Nal= nalidicic acid; Amp= ampicillin; Str= streptomycin; Chl= chloramphenicol; Kan= kanamycin; Neo= neomycin, and Tet= tetracycline.

type- kan.neo.cip, 1 showed triple R type-amp.neo.cip, 5 isolates exhibited triple R type-amp.kan.neo, 2 isolates exhibited quadruple R type- amp.kan.neo.cip, another 1 showed R type-amp.nal.neo.cip, 2 poultry isolates manifested quintuple R type-amp.kan.nal.neo.cip, 1 showed quintuple R type-tet.amp.chl.kan.neo, while 1 showed sextuple R type-tet.amp.kan.nal.neo.cip (Table 2). In terms of the mode of R determinant transfer 10/19 (53%) transferred the R determinant en bloc, seven, 7/19 (37%) did not transfer the resistance factor to the sensitive recipient *E. coli* K12, the other two, 2/19 (10%) transferred amp each from the initial amp.kan. neo exhibited by each of the two isolates, thus the resistance was not transferred *en bloc* but as segregation. None of the 19 poultry isolate transferred ciprofloxacin resistance (Table 3). Three of the poultry isolates, 3/19 (15.8%) transferred tetracycline resistance, 10/19 (52.6%) transferred ampicillin resistance, 6/19 (31.6%) transferred kanamycin resistance, 10/19 (52.6%) transferred neomycin resistance, 2/19 (10.5%) transferred resistance for nalidixic acid. None of the isolate transferred ciprofloxacin resistance. Two cattle *S. enterica* isolates 2/24 (8%), manifested 1 quadruple R type-tet.amp.kan.neo and 1 sextuple R type tet.amp.chl. kan.neo.cip (Table 2). The two cattle isolates transferred the R determinants en bloc as R type-tet.amp.chl. kan.neo.cip, tet.amp.kan.neo. One of the cattle isolate transferred the ciprofloxacin resistance (Table 3). The two cattle isolates 2/2 (100%) transferred the resistance factors for, tetracycline, ampicillin, kanamycin, neomycin, however, only one isolate, 1/2 (50%) transferred the R factors for chloramphenicol and ciprofloxacin. In Nigeria, Adetosoye (1980), reported that *S. glostrup* transferred CHL and STR resistant determinant, *S avonmouth* strains transferred STR resistance determinant, to sensitive *E coli* K12 recipients. The strains of *S labadi* (from fowls), *S. Dublin* (from cow), *S virchow* (from cow), transferred their R determinants to sensitive *E. coli* K12 recipients. The *S.* Typhimurium phage type 29 from intensively reared calves flock was associated with an outbreak in the Great Britain which resulted human casualties because the organism was resistant to chloramphenicol, the then drug of choice for the treatment of enteric fever in human (Anderson, 1969; Swann 1969). Likewise in the 1990s, a strain of *S.* Typhimurium resistant to ampicillin, chloramphenicol, streptomycin, sulphonamide and tetracycline (R type ACSSUT emerged in the United States and Europe's (Glynn et al., 1998).

The 2 lizard *S. enterica* isolates 2/24 (8%) showed 1 quintuple R type- tet.amp.kan.neo.cip and 1 septuple R type-tet.amp.chl.kan.nal.neo.cip. Also the two lizard isolates

Table 3. Antibiotic resistance transfer mode by transconjugants of the *S. enterica* isolates from animal sources.

S. enterica isolate code	Antibiotic transferred	Form of transfer
A4	Tet.Amp.Kan.Neo	*En bloc*
A5	Kan.Neo	*En bloc*
A6	Amp.Nal.Neo	*En bloc*
A7	Nal.Neo	*En bloc*
B10	Tet.Amp.Kan.Neo	*En bloc*
B15	Tet.Amp.Chl.Kan.Neo.Cip	*En bloc*
A24	Tet.Amp.Kan.Neo	*En bloc*
A28	Tet.Amp.Kan.Neo	*En bloc*
A29	Amp.Neo	*En bloc*
A30	Nt	NT
A31	Amp.Kan.Neo	*En bloc*
A32	Nt	NT
A33	Amp	Segregation
A34	Amp	Segregation
A35	Nt	NT
A36	Nt	NT
A37	Amp.Kan.Neo	*En bloc*
A38	Amp.Neo	*En bloc*
C40	Tet.Amp.Kan.Neo	*En bloc*
C41	Tet.Amp.Chl.Kan.Nal.Neo	*En bloc*
A43	NT	NT
A44	NT	NT
A45	NT	NT

Cip= Ciprofloxacin; Nal= nalidicic acid; Amp= ampicillin; Str= streptomycin; Chl= chloramphenicol; Kan= kanamycin; Neo= neomycin, and Tet= tetracycline.

Table 4. Reconstitution with different concentration of antimicrobial agents (µg/ml) of MacConkey agar for use as selective media.

S/N	Medium	Cip	Nal	Amp	Strep	Chl	Kan	Neo	Tet
1	A	-	-	-	100	-	-	-	-
2	B	50			100				
3	C		50		100				
4	D			50	100				
5	E				100	50			
6	F				100		50		
7	G				100			50	
8	H				100				50

Cip= Ciprofloxacin; Nal= nalidicic acid; Amp= ampicillin; Str= streptomycin; Chl= chloramphenicol; Kan= kanamycin; Neo= neomycin, and Tet= tetracycline.

transferred the resistance factors *en bloc* as R types-tet.amp.chl.kan.nal.neo and tet.amp.kan.neo. The two lizard isolates, 2/2 (100%) transferred the resistance factors for tetracycline, ampicillin, kanamycin, neomycin, whereas only one isolate, 1/2(50%) transferred for chloramphenicol and nalidixic acid. The occurrence of multidrug resistant *S. enterica* isolates in lizards from poultry houses indicates a reservoir for poultry salmonellosis. Geue and Löschner, (2002); Pasmans et al., (2005) earlier documented that reptiles are identified reservoirs of *Salmonella* spp. and the number of reports about reptile-associated salmonellosis is increasing. As observed in this work, *S. enterica* strains from food animals and lizards are showing increasing antimicrobial resistance and multiple resistance profiles (Threlfall, 2002; Hakanen et al., 2005). This could be attributed to gross misuse and

abuse of antibiotics in poultry and food animal in some parts of Nigeria. For example, Ogunleye et al. (2008) had earlier reported occurrence of multidrug resistant *E. coli* of poultry origin in Abeokuta, Ogun State due to abuse and misuse of antibiotic. The result of the current investigation is a further confirmation of the need for prudent and judicious use of antibiotics in the food animals to curtail the incidence of drug resistance and infective drug resistance transfer among enteric pathogens so as to prevent the spread of untreatable salmonellosis. It is relevant to note that all the poultry *S. enterica* isolates were from dead birds. Ciprofloxacin is the current drug of choice for treatment of enteric fever in human worldwide (Bhan et al., 2005). The occurrence of ciprofloxacin resistance transferred in one of the cattle isolate is of great public health concern, since the *S. enterica* was isolated from non-diarrhoeic cattle. Nationwide antibiotic resistance surveillance among food animals covering all the geopolitical zones in Nigeria is recommended to ascertain the current status of antimicrobial resistance in the nation. There is need to formulate scientific policy on containment of spread of antimicrobial resistance *vis-à-vis* judicious use of antibiotics among food animals as well as the possible spread of resistance from animal to human.

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