Plasmids: A Vehicle for Rapid Transfer of Antibiotic Resistance Markers of *Salmonella* Species in Animals

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ABSTRACT: This study was carried out to determine the prevalence of *Salmonella* transferable plasmids mediated antibiotics resistance in broiler chickens and diarrhea patients in Benin City, Edo State, Nigeria. A total of 183 *Salmonella* species were isolated from 1320 fecal samples of diarrhea patients and poultry chickens and tested for antibiotics resistance transferable ability. The rapid alkaline DNA extraction procedure was used to screen for the plasmids resistance markers. Seventy eight (42.6%) of the isolates were resistant to three or more antibiotics. The isolates were highly resistant to ampicillin, chloramphenicol, gentamicin, trimethoprim, tetracycline and sulfamethoxazole, and to a lesser extent, resistant to ciprofloxacin, ofloxacin, ceftriaxone and cefuroxime. Thirty two (17.5%) of the isolates had plasmids of varying sizes ranging from 2.5kb to 5.0kb while 151 (82.5%) appear to have no plasmids. These plasmids were highly transferable at a frequency of 2×10^{-2} to 4×10^{-4} per donor cell by conjugation. The exchange of plasmid(s) between bacterial cells and the integration of resistance genes into specialized genetic elements play a major role in acquisition and dissemination of antibiotic resistance genes among the *Salmonella* species. The results also demonstrated how antibacterial treatment targeted at a pathogenic organism in a host may affect the endogenous flora of another host in a population. The transfer of antibiotic resistant genes among the species is an increased risk considering the fact that all strains of this organism are potential pathogens. [The Journal Of American Science. 2007;3(4):86-92]. (ISSN: 1545-1003).

Keywords: *Salmonella*, Plasmids Resistance Transfer Markers.

INTRODUCTION

Salmonella infections are the most common widespread cause of salmonellosis among humans and animals worldwide, causing an estimated 1.41 million cases of infections and 500 human deaths annually in the United States of America (Blaser, 1996; Mead *et al.,* 1999). Animals used for food production often carry *Salmonella*, thus contaminating meat, dairy products, and eggs (Baird-Parker, 1990; Osterom, 1999). It is however difficult to evaluate the extent of infections in developing countries because of the very limited scope of studies and lack of coordinated epidemiological surveillance systems (Santos *et al.,* 2003).

Selective pressure imposed by the use of antimicrobials in both human and veterinary medicine promotes the spread of multiple antimicrobial resistances resulting in the growing problem of infections that are difficult to treat (Carattoli, 2003). Resistance to some ß-lactam antibiotics, tetracyclines, chloramphenicol, or trimethoprim is reported with increasing frequency (Gallardo *et al.,* 1999 and Velonakis *et al.,* 2001). The adverse effect of antimicrobial resistance has typically been recognized as treatment failure; the disease caused by the pathogen can significantly worsen because of the antimicrobial drug used. Evolution of plasmids through the acquisition of resistant genes had been reported, describing novel mechanisms for short-term accumulation of resistance determinants in plasmids circulating in *Salmonella* (Threlfall, 2002; Carattoli, 2003). There is no documented study on the antibiotic transferability pattern among *Salmonella* species in broiler chickens and patients in Nigeria. This study was therefore designed to investigate the frequency of transfer of plasmid-mediated resistance to antibiotics, widely used for treatment, among *Salmonella* species of human and poultry origin in Nigeria.

MATERIALS AND METHODS

Sample collection and Isolation of bacteria:

Samples were collected from feces of diarrhea patients and feces from 6 reputable broiler poultry farms. A total of 121 *Salmonella* species were obtained from 920 fecal samples of diarrhea patients from Central Hospital Benin City while 62 *Salmonella* species were obtained 400 fecal samples of broiler poultry chickens in Benin City. All the samples were cultured into selenite F broth in screw-capped bottles and incubated at 37 ^{0}C for 3-5 days. Subcultures were then made into plates of deoxychocolate citrate agar, and incubated for another 24 hours. Colonies with black centers were sub-cultured onto nutrient agar and incubated for another 24 hours. The cultures on nutrient agar plates were subjected to Gram-staining, motility, urease production, hydrogen sulfide production and citrate utilization tests. All Gram-negative, rod-shaped, motile, urease-negative isolates that produced acid on triple sugar Iron agar slants and able to utilize citrate as sole carbon source were identified as species of the genus *Salmonella*.

Antibiotic Susceptibility Testing:

The E-test method (AB Biodisk) was used to screen for the antibiotic susceptibility patterns. The minimum inhibitory concentration (MIC) susceptibility test was determined in accordance with the manufacturer's guidelines (AB Biodisk, Sweden). The 0.5 McFarland standards isolates were inoculated onto Mueller Hinton agar plates by swabbing evenly in three directions. The E-test strip (obtained from the refrigerator at 4^{o}C) was applied to each plate with sterile forceps with lowest concentration toward the center of the agar plate. The plates were then incubated at 30 to 35 °C for 24 hours. The E-test MIC values were read directly from the E-test strip MIC scale. The following antibacterial agents: ofloxacin (Ofl), ciprofloxacin (Cip), cefuroxime (Cef), ceftriaxone (Ce), gentamicin (Gn), trimethoprim-sulfamethoxazole (Txm-Sal), ampicillin (Am), chloramphenicol (Chl) and tetracycline (Te) were used. The concentration gradient of each antimicrobial agent on the E-test strips was 0.016 to 256µg/ml with the exception of ciprofloxacin and ofloxacin for which the gradient ranged from 0.002 to 32µg/ml. The susceptibility range as defined by AB Biodisk Sweden were: ofloxacin (S $\leq$2, I = 4 and R $\geq$ 8), ciprofloxacin (S $\leq$1, I = 2 and R $\geq$ 4), cefuroxime (S $\leq$8, I = 16 and R $\geq$ 32), gentamicin (S $\leq$4, I = 8 and R $\geq$ 16), trimethoprim-sulfamethoxazole (S $\leq$2 and R $\geq$ 4), ampicillin (S $\leq$8, I = 16 and R $\geq$ 32), chloramphenicol (S $\leq$8, I = 16 and R $\geq$ 32), ceftriaxone (S $\leq$8, I = 16 and R $\geq$ 32) and tetracycline (S $\leq$4, I = 8 and R $\geq$ 8) where S = sensitivity, I = intermediate and R = resistance.

Conjugation and Plasmids profiles:

Conjugation experiments were performed as described by Yukata *et al.* (2004) and Wang *et al.* (2004) using *E coli* strains obtained from Nigerian Institute for Medical Research (NIMR), Lagos, as recipient. The donors and recipients-plasmid -free - rifampicin resistant strains were incubated both on Mueller Hinton broth culture (Difco Laboratories Detroit, Mich USA) and on Oxoid Mueller Hinton agar (Difco Laboratories Detroit, Mich USA) at 37^{0}C for 18 hours. The transconjugants were selected on Mueller Hinton agar medium supplemented with 200µg/ml rifampicin (Daiichi Pharm. Co. Ltd, Japan) to inhibit the growth of the donor and recipient respectively. The frequency of transfer of the plasmids were determined by dividing the number of transconjugants by the number of donor cells according to Wang *et al.* (2004) and Yukata *et al.* (2004). The transconjugants were re-streaked onto fresh selective culture plates and their identities were re-confirmed on the basis of their biochemical methods and their antibiotics resistance pattern re-confirmed. The Birnboim and Doly (1979) method was employed for screening plasmids (rapid alkaline extraction) of donors and transconjugants. The plasmids DNA were then electrophoresed on 0.8% agarose gel, stained with 14µl ethidium bromide. The DNA was then photographed with Polaroid camera and viewed using UV trans-illumination. The molecular weights and distances were then determined using standard methods according to Meyers *et al*. (1982) and Birnboim and Doly (1979) with standard DNA molecular weight marker II (0.12-23.1kbp) of bacteriophage lambda HindIII (Roche Diagnostic GmbH).

Statistical analysis:

The MIC values were compared using the Chi-square test and the student two-tailed t test. A difference was considered significant when P-value by the two-tailed was less than 0.05 ($P<0.05$). Results were expressed as mean standard deviation ($\overline{\times} \pm S.D$).The calculated values where then

compared with the critical values at the appropriate degree of freedoms at a significant level of P= 0.05 (21).

RESULTS

The results revealed that 121 and 62 *Salmonella* species respectively were obtained from 920 fecal samples of diarrhea patients and 400 fecal samples of broiler poultry chickens (Table 1). Out of the 183 *Salmonella* species, 78 were resistant to three or more antibiotics. The isolates from both sources were highly resistant to ampicillin, chloramphenicol, gentamicin, trimethoprim-sulfamethoxazole and tetracycline. The prevalence of resistance in all the groups was very low to ciprofloxacin, ofloxacin, ceftriaxone and cefuroxime. There was no significant difference ($P<0.05$) between the resistance patterns of the species from patients and broiler chickens. Apart from *Salmonella species*, other bacterial isolates encountered were *E. coli, Shigella, Pseudomonas aeruginosa, Enterococcus, Klebsiella* and *S. aureus*,.

The results of the plasmids analyses show that 32 (17.5%) had plasmids of varying sizes ranging from 2.5kb to 5.0kb while 151 (82.5%) appear to have no plasmids (Table 2). These plasmids were highly transferable at a high frequency of 2×10^{-2} to 4×10^{-4} per donor cell by conjugation.

Table 1: Percentage (%) Occurrence of resistant *Salmonella* species from diarrhea patient's and from broiler feces

	Percentage of resistant isolates to antibiotics								
Source	**Cip**	**Ofl**	**Te**	**Am**	**Txm-sal**	**Chl**	**Gn**	**Cef**	**Ce**
Diarrhea patients n= 121	8(6.6%)	4(3.3%)	67(55.4%)	92(76.0%)	58(47.9%)	103(85.1%)	59(48.8%)	7(5.8%)	8(6.6%)
Broiler chickens n = 62	5(8.1%)	3(4.8%)	49(79.0%)	46(74.2%)	37(59.7%)	32(51.6%)	27(43.6%)	2(3.2%)	3(4.8%)

Key: Ofloxacin (Ofl), ciprofloxacin (Cip), cefuroxime (Cef), ceftriaxone (Ce), gentamicin (Gn), tetracycline (Te), Trimethoprim-sulphamethoxazole (Txm-Sal), ampicillin (Am) and chloramphenicol (Chl)

DISCUSSION

The routine use of antibiotics in medicine and agriculture circles has resulted in widespread antibiotic resistance and in the development of genetic mechanisms efficient for the dissemination of antibiotic genes, especially, within and between species of microorganisms. These uncontrolled uses of antimicrobials in agriculture and for treating human patients contribute to increase multi-drug resistance of *Salmonella* species. The present results showed that 121 *Salmonella* species were obtained from human sources while 62 species were obtained from broiler chickens. This high prevalence of *Salmonella* species in both sources highlights the need to monitor the spread of the microorganism through animal products. The high percentage of broiler chickens contaminated with *Salmonella* was higher than previous reports (Morgan, 1980; Velonakis *et al.,* 2001; Threlfall, 2002; Guncagu, 2004). The contaminating of microorganisms in poultry products are reportedly derived from the poultry manure, poultry workers, equipment, poultry's environment which include faecal, soil and water. Many of the outbreaks reported have been epidemiologically linked to the consumption of raw or undercooked eggs, and to a lesser extent, chicken (Hedberg *et al.,* 1993; Araújo *et al.,* 1995; Bangtrakulnonth *et al.,* 2004). The frequent occurrence of *Salmonella* species in chicken suggests that poultry may be an important reservoir, a finding that is consistent with almost all other studies in other countries. Moreover, *Salmonella* infections have been recognized as a major public health concern both from developed and developing countries (Helms *et al.,* 2004; 2005).

Secondly, *Salmonella* is widespread in nature and can colonize or infect a variety of domesticated and wild animals ranging from mammals to birds and reptiles. According to Winokur *et al.* (2000), most human *Salmonella* infections in the United States are related to ingestion of contaminated food products rather than person-to-person transmission or direct fecal-oral transmission. Many outbreaks have been traced to ingestion of contaminated animal products and in some cases, traced to specific farms, flocks, or herds of animals (Altekruse *et al.,* 1993). According to Matofari *et al.* (2007) healthy camels can be carriers of *Salmonella* species which can be isolated from their faeces and lymph nodes on slaughter of camels. Camels that are chronic carriers of *Salmonella* may present a human health hazard through consumption of camel products like milk (Matofari *et al.,* 2007).

Table 2: Antibiotics Resistant Pattern of *Salmonella* species Transconjugants

Code of Isolates	Source	Resistant Marker Spectrum of Donors	Plasmids Profile of Donors (kb)	Plasmids Profile of ransconjugants (kb)	Resistant Marker Spectrum of transconjugants
DB12	Diarrhea	Am,Chl,Txm-Sal,Gn, Te	2.5, 4.3, 5.0	4.5, 4.3	Am^r,Te^r,Chl^r
BB15	Broiler	Am,Gn,Chl,Txm-Sal, Te,Ofl,	4.5, 4.7	4.5, 4.7	$Am^r$$Gn^r$,$Te^r$, Txm-$Sal^r$
BB16	Broiler	Txm-Sal,Te,Am,Ce	2.5, 4.3, 5.0	4.3, 5.0	Txm-Sal^r, $Te^r$$Am^r$
DB18	Diarrhea	Am,Chl,Gn,Ofl Te	2.5, 4.3, 5.0	4.3, 5.0	Am^r,Chl^r,Gn^r,Te^r
BB01	Diarrhea	Am,Gn, Te,Cef	2.5, 4.3, 5.0	4.3, 5.0	Am^r,Gn^r,Te^r
BB23	Broiler	Am,Txm-Sal, Te,Ofl,	2.5, 4.3, 5.0	4.3, 5.0	Am^r,Txm-Sal^r, Te^r
DB18	Broiler	Am,Chl,Txm-Sal, Te,Cip	2.5, 4.3, 5.0	4.3, 5.0	Am^r,Chl^r, Txm-Sal^r,Te^r
DB213	Diarrhea	Am,Gn,Te,Ofl	2.5, 4.3, 5.0	4.3, 5.0	Am^r,Gn^r,Te^r
BB145	Broiler	Am,Gn,Te,Ofl	2.5,3,4. 7,	2.5, 4.5, 4.7	Am^r,Gn^r,Te^r
DB87	Diarrhea	Am,Chl,Gn,Ofl, Te	2.5,4.3, 4.7, 5.0	4.5, 4.7	Am^r,Chl^r,Gn,Te^r
DB92	Diarrhea	Am,Gn,Chl,Cip	2.5, 4.3, 5.0	4.5, 4.7	Am^r,Gn^r,Chl
DB108	Diarrhea	Am,Chl,Txm-Sal, Te	2.5, 4.3, 5.0	4.5, 4.7,5.0	Am^r,Chl^r, Txm-Sal^r, Te^r
BB211	Broiler	Am,Gn,Te,Txm-Sal, Ofl,	4.5, 4.7 4.3, 5.0	4.5, 4.7,5.0	Am^r,Gn^r, Txm-Sal^r
BB266	Broiler	Am,Txm-Sal, Te,Ofl,	2.5, 4.3, 5.0	4.5, 4.7,5.0	Am^r,Txm-Sal^r, Te^r
DB59	Diarrhea	Am,Chl,Gn,Ofl,Te	2.5, 4.3, 5.0	4.5, 4.7,5.0	Am^r,Chl^r,Gn^r, Te^r
BB178	Broiler	Am,Gn,Te,Cip,Ce	4.5, 4.74.5, 4.7,5.0	4.5, 4.7,5.0	Am^r,Gn^r, Te^r,Cip^r
BB283	Broiler	Gn,Chl,Txm-Sal, Te,Ofl	4.5,4.7,4.5, 4.7,5.0	4.5, 4.7,5.0	Gn^r,Chl^r, Txm-Sal^r, Te^r
DB65	Diarrhea	Am,Txm-Sal,Te, Ofl, Gn	4.5, 4.7, 4.5, 4.7,5.0	4.5, 4.7,5.0	Am^r,Txm-Sal^r, Te^r,Gn^r
DB59	Diarrhea	Chl,Gn,Ofl,Te,Am	2.5, 5.0	5.0	Gn^r,Te^r,Am^r

Key: Ofloxacin (Ofl), Ciprofloxacin (Cip), Cefuroxime (Cef), Ceftriaxone (Ce), Gentamicin (Gn), Tetracycline (Te), Trimethoprim-sulphamethoxazole (Txm-Sal), Ampicillin (Am), Chloramphenicol (Chl), BB = Broiler Chickens Transconjugants and DB = Diarrhea Patients Transconjugants.

The present results raises the concern that there may be a link among antibiotic use in feeds, the development and presence of antibiotic resistance among bacteria in food-producing animals, and antibiotic associated bacterial infection in humans. This is because all the *Salmonella* isolates from both sources used in this study showed resistance to the entire antibiotic tested. Overall, *Salmonella* isolates in this study showed high resistance to a number of antibiotics, ranging from 3.3% in human feces to 85.1% as compared to *Salmonella* isolates in broiler chickens that ranged from 3.2% to 79.0%. There was no significant different ($P>0.05$) between the antibiotics resistance pattern from the two sources. This high antibiotic resistance rates could be due to the widespread use of antibiotics in chickens, particularly in feed, as well as their indiscriminate use. The results showed a high level of *Salmonella* resistance to tetracycline in the birds than the quinolones. There are evidences to indicate that tetracycline survives longer in the environment than do other antibiotics which may be critical in maintaining the level of tetracycline resistance at a high level (Frost, 1991). The low level of resistance to quinolones may be because they are relatively new antibiotics and are also more expensive than the tetracycline, ampicillin and chloramphenicol. There is also a probability that enteric bacilli in chicken intestinal tracts are not necessarily selected by antimicrobial supplementation of the feed, but rather on their common presence in the environments from which they can colonize the intestinal tracts of newly hatched chicks. These resistant enteric bacilli proliferate in the intestine and may transfer their resistance to *Salmonella* and from there to human. Because of this, the committee of the Joint Expert Advisory Committee on Antibiotic Resistance in Australia (JETACAR, 1999), agreed that there was evidence for the emergence of resistant bacteria in human and animal following antibiotic use and the spread of resistant bacteria from animal to

human as well as the transfer of antibiotic-resistant genes from bacteria in animal to human pathogens and that strains of resistant bacteria which are zoonotic can cause disease in human (JETACAR, 1999).
The high antibiotic resistance demonstrated by these isolates correlated with the high level antimicrobial resistance in enterobacteria, in fecal flora as well as in clinical isolates reported by Aarestrup, (1999) and Velonakis *et al.* (2001). The isolates were highly resistant to tetracycline, chloramphenicol, gentamicin, trimethoprim-sulfamethoxazole and ampicillin and less resistance to ciprofloxacin, ofloxacin, ceftriaxone and cefuroxime from both sources. This also confirms the idea that uncontrolled sale and use of antimicrobials in agriculture and for treating human patients could contribute to increase multi-drug resistance of *Salmonella* species. There is the potential for antibiotic-resistant *Salmonella* to spread through the food chain from animals treated with antibiotics to humans.

There are several reports on the trend of infection through feces, suggesting that *Salmonella* species and *Escherichia coli* are the main carrier of antimicrobial resistance genes in fecal flora of birds and human (Hedberg *et al.,* 1993; Araújo *et al.,* 1995; Carattoli 2003; Helms *et al*., 2005; Osman *et al.,* 2006). Resistance genes are often located on extra-chromosomal genetic elements or in segments inserted within the chromosome that originates from other genomes (Carattoli, 2003; Yah *et al.,* 2007). The acquisition of a new gene may occur by genetic transformation or through mobilization by conjugative transfer. The latter may occur at high frequency and efficiency, and several resistance genes can be acquired simultaneously (Carattoli, 2003).

The results showed that 32 (17.5%) of the isolates had plasmids of varying sizes ranging from 2.5kb to 5.0kb while 151 (82.5%) appear to have no plasmids. According to Carattoli (2003) and Yah *et al.* (2007), the antibiotic resistance in those isolates which seem not to possess plasmids was associated with chromosome and/or transposons instead of being plasmid-mediated. This therefore implies that there is no consistent relationship between antibiotic resistance pattern and the number of plasmid bands present. Conjugation studies were performed to determine whether the plasmids resistance markers could be transferred to *E. coli*. The results showed that all the transconjugants expressed plasmid DNA that migrated approximately on agarose gels. These plasmids were highly transferable at a frequency of 2×10^{-2} to 4×10^{-4} per donor cell. The transferred plasmids DNA varied among the *Salmonella* species. Transfer was higher among ampicillin (Am^r), tetracycline (Te^r) gentamicin (Gn^r) resistance genes while ofloxacin (Ofl^r), ciprofloxacin (Cip^r), cefuroxime (Cef^r), and ceftriaxone (Ce^r), were not transferred at all. The exchange of plasmid(s) between bacterial cells and the integration of resistance genes into specialized genetic elements play a major role in acquisition and dissemination of antibiotic resistance genes among the *Salmonella* species (Winokur *et al.,* 2000; Carattoli, 2003; Helms *et al.,* 2004; Osman *et al.,* 2006; Yah *et al.,* 2007). We suggested that the multiple drug resistance of *Salmonella* in broiler chickens may be transferred from animal strains to the resident flora of the human gut. Such transfer could occur during transient passage through the digestive tract. The results also, demonstrated how antibacterial treatment targeted at a pathogenic organism in a host may affect the endogenous flora of another host in a population. The transfer of antibiotic resistant genes among the species is an increased risk considering the fact that all strains of this organism are potential pathogens.

CONCLUSIONS

The present study support the hypothesis that the versatility of plasmids together with the usage of antimicrobials in human and birds, may largely contributed to the spread of antimicrobial resistance. Also the presence of antibiotics resistant genes in *Salmonella* can be explained by horizontal and vertical transfer of resistance from bacteria of nosocomial origin. This phenomenon is a disturbing development for public health, since *Salmonella* carriage of such transmissible plasmids may facilitate the spread of a variety of resistance. The transfer of such a multi-resistance gene fragment to other pathogenic bacteria could result in a serious health concern. An understanding of the antibiotic resistance gene arrangements will probably have an appreciable impact on antibiotic use in agriculture and medicine.
Salmonella species is a public health risk. All strains of this organism are potentially pathogens. The transfer of antibiotic resistant genes among the species is an increased risk.

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