A Survey of the Antibiotic Resistance of Human and Poultry Strains of Salmonella from Southwest Nigeria.

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Abstract: The high antibiotic usage in poultry is a public health concern, especially in South-west Nigeria. The human clinical isolates obtained from UCH Ibadan and NIMR Lagos was sensitive to nitrofurantoin, gentamicin, and nalidixic acid, and tetracycline except in few cases. Only 8(32%) isolates showed susceptibility to amoxicillin. Analysis showed that 15(15%) human isolates were resistant to cotrimoxazole. Only 15(60%) showed resistance to chloramphenicol, while erythromycin and cloxacillin showed no activity against all the human isolates. The poultry isolates were resistant to almost all the antibiotics tested. However, ofloxacin a fluoroquinolone showed activity to all the poultry isolates, while 4(16%) isolates were resistant to gentamicin. Though the MIC of the isolates were not detected, out of thirty-nine *Salmonella* isolates selected based on their resistance to Augumentin, only five isolates were found to be β-lactamase positive. Plasmid DNA having molecular weights ranging from 11,561bp to 13,083bp was isolated from six strains.

Introduction

The bacterium classified as *Salmonella enterica* is capable of infecting a variety of hosts, resulting in diseases ranging from self-limiting gastroenteritis to life-threatening systemic infections. *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) causes disease in mice and humans, yet the nature of the infection is distinctly different in each host (Prescott, et al.,1991).

Typhoid fever, one of the infections caused by *Salmonella enterica* serovar Typhi is rated the second most common infection of *Salmonella* (Hirose et al., 2001). It is an invasive, systemic infection that is caused by the ingestion of *Salmonella* Typhi either in food or water (Prescott, et al., 1991). Poultry meat and eggs represent an important source of human infection with *Salmonella* spp. (Campell, and Gilbert, 1995).

Consequently, the indicriminate use of antibiotics can select not only for resistance in pathogenic bacteria (Prescott, *et al*.,1991). In most developing countries, the most available antibiotics are chloramphenicol, ampicillin and cotrimoxazole, with chloramphenicol being the drug of choice for more than forty years and ampicillin, cotrimoxazole, trimethroprim-sulfamethoxazole and amoxycillin being effective alternatives (Hirose et al., 2001). In Nigeria, as in most tropical countries, MDRST is also high. In a study carried out by Olukoya *et al*., (Olukoya et al., 2000). Numerous outbreaks of infections with *Salmonella* producing extended spectrum β-lactamases have been observed worldwide which has posed a serious threat to the use

of many classes of antibiotics especially the cephalosporins (Palucha et al., 1999). They were identified after the introduction of the extended spectrum β-lactams (Bradford, 2001). In any case, report has it that, ESBLs in Gram-negative bacteria are encoded by transferable conjugative plasmids which also code resistant determinants to other antibiotics such as aminoglycosides, tetracycline, chloramphenicol and the flouroquinolones (Soge et al., 2006). Although, many of the Gram-negative bacteria like *Salmonella*, possess a naturally occuring chromosomally-mediated β-lactamases (Jacoby and Munoz-Price, 2005). Resistance is often associated with the acquisition of mobile genetic elements that carry one or more drug resistance genes (Schwarz and Danclab, 2001). These mobile genetic elements can be plasmids, transposons or gene cassettes/integrons (Ojo, 2002). In *Salmonella*, the wild type strains are known to be plasmid carriers (Georges-Courbot, 1990). Plasmid profiling in epidemiological typing is hinged on the fact that isolates belonging to a particular epidemic strain will have identical plasmid content and so their profile will be identical (Threlfall, 1992). However, the use of antimicrobial agents in animal feeds as prophylactic, chemotherapy, and growth promoters especially in poultry has been a major factor in the emergence of *Salmonella* with decreased susceptibility to antibiotics (Threlfall, 1992). Thus, in this study, it was thought necessary therefore, to verify the susceptibility pattern in human and poultry, the causes and the possibility of the negative impacts on both respectively, especially in southwest Nigeria.

Material and methods
Collection of sample and Antimicrobial suscepibility test

Twenty-five Human clinical isolates were obtained from routine section of Medical Microbiology Laboratory, University College Hospital (UCH), Ibadan, and Nigerian Institute of Medical Reseach (NIMR), Yaba, Lagos. Also the twenty-five poultry isolates of *Salmonella* spp. were obtained from Zartech Farm Laboratory (ZFL) Ibadan between April – June, 2011. Commercially used antibiotics (Abteck) were used on the test isolates adopting the method of disc diffusion. A dilution of 0.1ml of 10^{-2} dilution of an overnight broth culture of each test bacterium was put into each Petri-dish aseptically, and 20ml molten agar was added followed by gentle rotation of the plate for uniform mixing. The mixture was then allowed to set. The antibiotic discs were applied on the surface of the seeded agar plates such that the centres were 25mm apart as designed by the manufacturers. The plates were incubated at 37°C for 18 hours after about 15 minutes of applying the discs. The plates were then examined for zones of growth inhibition in millimetre (mm) (CLSI,2000).

Detection of β-lactamase

The cell-suspension method (Iodometric method) was employed (Adeleke and Odelola, 1997). Overnight nutrient broth culture of each isolate was subcultured by streaking on nutrient agar plate and incubated at 37°C for 24 hours. Bacterial cell suspension was prepared in triplicate for each isolate by emulsifying bacterial colonies with a sterile wire-loop in 0.5ml of freshly prepared phosphate buffered solution containing penicillin G (0.06mg per ml). Briefly, the suspension in small sterile test-tubes was homogenized on a vortex mixer. An ordinary penicillin G phosphate buffered solution served as control. The test and control tubes were incubated at room temperature of 28°C for a an hour. Thereafter, two drops of freshly prepared 1% aqueous starch solution were added to each suspension. The mixture was gently shaken briefly, after which a drop of iodine solution was added to each tube without shaking the mixture. The tubes were allowed to stand at room temperature for 10 minutes. A colour change from blue/blue black to colourless indicate positive. No colour change within 10 minutes indicates negative results.

Plasmid DNA profiling, isolation and molecular weight Determination

Pure isolates were inoculated on Mueller Hinton broth and incubated overnight. The grown cells were harvested and suspended in 200µl of solution A {100 mM glucose-50 mM Tris hydrochloride (pH 8)-10 mM EDTA}. 400µl of freshly prepared 1% sodium dodecyl sulfate in 0.2 N NaOH was added and the samples were mixed by inverting tubes. 300µl of a 30% potassium acetate solution (pH 4.8) was added and the samples were mixed by vortexing. After incubating on ice for 5 minutes, the debris was removed by a 5-minute centrifugation in a centrifuge (model 5415R; Eppendorf). The supernatant was removed and extracted once with a phenol-chloroform mixture (1:1) and precipitated with an equal volume of isopropanol. The plasmid DNA was then dissolved in 50µl of TE buffer. A DNA molecular weight marker was also loaded into one of the wells. The gel electrophoresis was carried out on a 0.8% agarose gel with Tris-Borate-EDTA (TBE) buffer, in a horizontal tank at a constant voltage of 60°C for about 1 hour 30 minutes. After electrophoresis, plasmid DNA bands were viewed by fluorescence of bound ethidium bromide under a short wave ultraviolet light transilluminator and the photograph were taken using a photo documentation system.

Results and Discussion

However, *Salmonella* infections especially Typhoid and Paratyphoid fevers are important infections in developing countries like Nigeria, but with the introduction of antimicrobial therapy such as chloramphenicol in 1948, the high mortality rate of 30% that was usually observed was reduced to about 1%, hence, making the treatment and management of these infections easy and attainable (Mirza, 1995). However most isolates used in this study appeared highly resistant to the first line antibiotics like; chloramphenicol, cotrimoxazole, and tetracycline.

From the cultural/biochemical characterization of the fifty isolates studied, four species were identified, *Salmonella* Typhi (12-isolates), *Salmonella* Paratyphi A (3- isolates), *Salmonella* Paratyphi B (2- isolates), and *Salmonella enterica* (33- isolates) (Table 1).

Out of the twenty five human isolates tested against Augmentin, nine isolates showed sensitivity while sixteen showed resistance. Analysis showed that 15(15%) human isolates were resistant to cotrimoxazole. Out of the twenty five human isolates tested on Chloramphenicol, 15(60%) showed resistance. Erythromycin and cloxacillin had no activity against all the human isolates, while Amoxicillin, cloxacillin, and erythromycin showed no activity on poultry isolates (Table 2).

This study clearly showed that drug resistance among *Salmonella* isolates is quite high, especially among the poultry isloates. Similarly, most of the *Salmonella* isolates in this study showed multiple drug resistant (MDR), no isolate was resistant to less than four antibiotics among the human clinical

isolates and seven among the poultry isolates. Amongst the *Salmonella enterica* serovar Typhi isolates, all the isolates were multidrug resistant *S.* Typhi (MDRST) strains. i.e. they are resistant to three or more antibiotics. This high degree and widespread resistance was also observed by Olukoya *et. al*.(Olukoya et al., 2000).

In this study, 2(8%) resistance to nalidixic acid was observed in human isolates while 22(88%) resistance to nalidixic acid was observed in poultry isolates. Nalidixic acid is a quinolone that is very important in the therapeutic world of today, used to monitor the degree of resistance to fluoroquinolones (Ackers et al., 2000). Similarly in this study, only 1(4%) isolate resistance to ofloxacin a fluoroquinolone was observed in human isolates. Though previous studies have shown the emergence of strains that show reduced susceptibility to fluoroquinolones (Matsumoto et al., 1999). According to Das *et al*. (Das et al., 2000) they observed a 2.5% resistance to the fluoroquinolnes in Orissa, India. Thus, a 4% resistance observed in this study, shows the rising rate of drug resistance in Nigeria. However, in this study, none of poultry isolates, showed resistant to ofloxacin.   Again, fluoroquinolones has a rapid response to infections and they are cost effective when compared with the third generation cephalosporins and they still represent for multidrug resistant cases the drug of choice (Ackers et al., 2000).

A comparative look at the poultry isolates and the human isolates, results showed that the number of multidrug resistant strains amongst the poultry isolates was distinctively higher than the human strains. Only one human isolate (NIMR020N) showed resistance to nitrofurantoin, gentamicin, and nalidixic acid. A comprehensive examination of the results on Table 2 shows that 13(52%) of the poultry isolates were resistant to nitrofurantoin, while 1(4%) of the human isolates showed resistant to it. Similarly, 4(16%) of the poultry isolates were resistant to gentamicin, while 2(8%) of human isolates were resistant to it. All the human isolates tested against tetracycline showed susceptibility except isolate (NIMR011F). The same is applicable to nalidixic acid, where poultry isolates showed 22(88%) resistant as against 2(8%) in human isolates (Table 2). The resistance of both human and poultry isolates to cloxacillin and erythromycin may be attributed to the fact that they are mostly used on Gram-positive organisms. This susceptibility pattern can be attributed to the antimicrobial agents used in animals at subtherapeutic dose which result in increased transmission of resistant microorganisms between animals, and therefore result in increased transmission of *Salmonella* to humans through food.

For instance, when fluoroquinolones were first licensed for human therapy, no immediate rise in *Salmonella* resistance was observed. In contrast, when fluoroquinolones were subsequently licensed for use in food animals, the rates of fluoroquinolone-resistant *Salmonella* in animals and food, and then subsequently in human infections, rapidly increased in several countries (WHO, 1998). However, the emergence of MDR *S. enterica* serovar Typhi as detected in this study may be attributed to the irrational or indiscriminate use of antibiotics in treating human infections or in cases where they are likely to be of little or no therapeutic value. It could be due to the presence of plasmid DNA, also could be responsible for the transmission of antibiotic resistance through conjugation from other organisms like *E. coli* resident in a patient's intestinal tracts (Mirza et al., 1995). This practice has increasingly become the focus of concern as rresearchers have now found evidence that the use of antibiotics on farms has led to an increase in antibiotic-resistant cases (Kaufman, 2000).

In this study, though the MIC of the isolates were not detected, five isolates (*Salmonella spp.* UCH005, UCH018, ZFL021P, ZFL022P, and *Salmonella spp.* ZFL024P) produced β-lactamases out of the thirty-nine isolates that were selected for β-lactamase production test based on their resistance to Augmentin(Table 3). The five isolates are most likely to be resistant to β-lactam drugs like cephalosporins. Most ESBLs are typically plasmid-mediated rather than chromosomally- mediated β-lactamases [4, 9] as seen in this study. Plasmid-mediated extended spectrum   β-lactamases have been reported for *Salmonella*, but may also occur in other enteric Gram-negative bacteria (Bradford, 2001; Jacoby and Munoz-Price, 2005). However, a comparative look at the β-lactamase production (Table 3), and the plasmid profiling analysis results (Fig.1), respectively. Isolates (NIMR001N, NIMR011F, and NIMR020N) neither produced β-lactamase nor possess plasmids, yet, they were resistant to β-lactam antibiotics used in this study (Amoxicillin, Augmentin, Cloxacillin) and other antibiotics. This showed that they possess other resistance mechanisms other than that mediated by plasmid or β-lactamase production. These other resistance mechanisms which may have been employed by these isolates may include, chromosomally-mediated resistance, alteration in outer membrane proteins, inherent resistance, acquired resistance, endogenous resistance, or exogenous resistance.

In this study, out of nine *Salmonella* isolates that were profiled for the presence of plasmid, six *Salmonella* isolates carried plasmid, with molecular weight ranged from 11,561bp to 13,083bp. Similarly,

all the *Salmonella* strains carrying plasmid DNA in this study possessed only a single plasmid DNA, but its molecular weight varies among the resistant strains. Remarkably, all the selected strains from UCH Ibadan and ZLF Ibadan were the ones that bore plasmid while the selected strains from NIMR Lagos bore no plasmid even though they were multidrug-resistant. However, the resistant strains which did not posses plasmid DNA, could be an indication of chromosomally mediated bacterial drug resistance exhibited by these strains (Soge et al., 2006).

Table 1: The prevalence and percentage composition of *Salmonella* isolates from the sample collected:

Salmonella species	Number of isolates	Percentage composition (%)
S. Typhi	12	24
S. Paratyphi A	3	6
S. Paratyphi B	2	4
S. enterica	33	66

Table 2: Distribution of Antibiotic resistance/susceptibility level of the *Salmonella* isolates from Human and Poultry Clinical Isolates.

Antibiotics.	No. of Susceptible Human Isolates (%)	No of Resistant Human Isolates (%)	No. of Susceptible Poultry Isolates (%)	No of Resistant Poultry Isolates (%)
Ofloxacin	24(96)	1(4)	25(100)	0(0)
Nitrofurantoin	24(96)	1(4)	12(48)	13(52)
Gentamicin	23(92)	2(8)	21(84)	4(16)
Nalidixic acid	23(92)	2(8)	3(12)	22(88)
Tetracycline	22(88)	3(12)	4(16)	21(84)
Chloramphenicol	10(40)	15(60)	4(16)	21(84)
Cotrimoxazole	10(40)	15(15)	6(24)	19(76)
Augmentin	9(36)	16(64)	2(8)	23(92)
Amoxicillin	8(32)	17(68)	0(0)	25(100)
Cloxacillin	0(0)	25(100)	0(0)	25(100)
Erythromycin	0(0)	25(100)	0(0)	25(100)
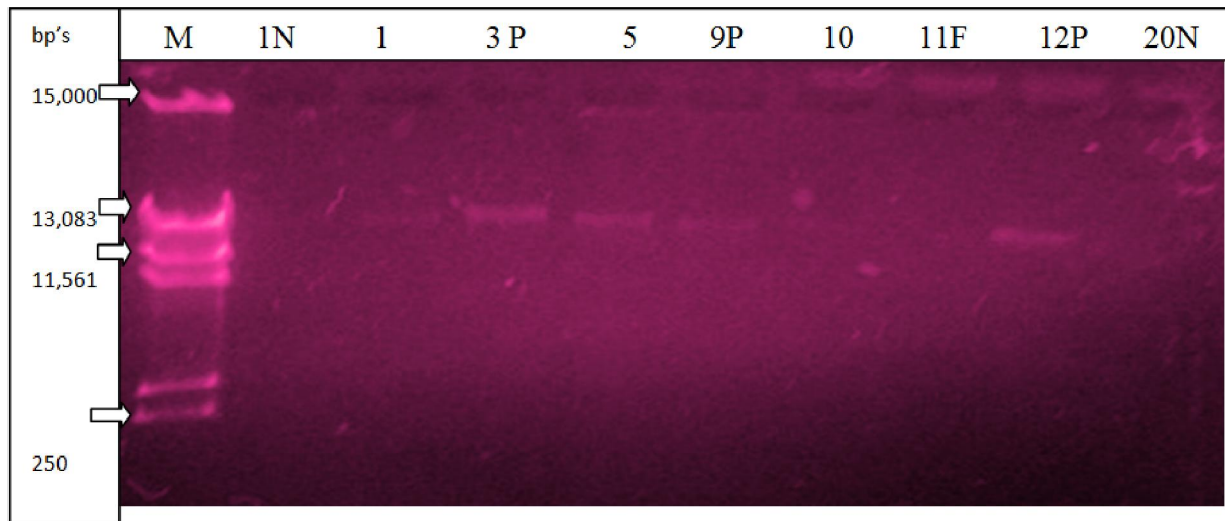
Fig 1: Agarose gel electrophoresis of the plasmid DNA in selected *Salmonella* isolates. Lane M shows HindII DNA molecular weight marker of 250bp's. Lane IN shows *Salmonella* NIMR001N (no plasmid), lane 1 *Salmonella* UCH001 having plasmid of molecular weight 12,298bp,lane 3P *Salmonella* ZFL003P (13,083bp), lane 5 *Salmonella* UCH005 (11,561bp), lane 9P *Salmonella* ZFL009P (11,561bp), lane 10 *Salmonella* UCH010 (11,561bp), lane 11F *Salmonella* NIMR011F (no plasmid), lane 12P *Salmonella* ZFL012P (11,561bp) and lane 20 N *Salmonella* NIMR020N (no plasmid)

Table 3: The level of β-lactamase produced by the Augmentin resistance *Salmonella* isolates.

Clinical Isolates	β-Lactamase Production/ Source	
Salmonella UCH001	-	Human
Salmonella UCH003	-	Human
Salmonella UCH004	-	Human
Salmonella UCH005	+	Human
Salmonella UCH010	-	Human
Salmonella UCH018	+	Human
Salmonella NIMR005F	-	Human
Salmonella NIMR011F	-	Human
Salmonella NIMR022F	-	Human
Salmonella NIMR025F	-	Human
Salmonella NIMR030F	-	Human
Salmonella NIMR033F	-	Human
Salmonella NIMR001N	-	Human
Salmonella NIMR020N	-	Human
Salmonella NIMR123N	-	Human
Salmonella NIMR170N	-	Human
Salmonella ZFL001P	-	Poultry
Salmonella ZFL002P	-	Poultry
Salmonella ZFL003P	-	Poultry
Salmonella ZFL004P	-	Poultry
Salmonella ZFL005P	-	Poultry
Salmonella ZFL006P	-	Poultry
Salmonella ZFL007P	-	Poultry
Salmonella ZFL008P	-	Poultry
Salmonella ZFL009P	-	Poultry
Salmonella ZFL010P	-	Poultry
Salmonella ZFL011P	-	Poultry
Salmonella ZFL012P	-	Poultry
Salmonella ZFL013P	-	Poultry
Salmonella ZFL014P	-	Poultry
Salmonella ZFL015P	-	Poultry
Salmonella ZFL016P	-	Poultry
Salmonella ZFL017P	-	Poultry
Salmonella ZFL018P	-	Poultry
Salmonella ZFL019P	-	Poultry
Salmonella ZFL020P	-	Poultry
Salmonella ZFL021P	+	Poultry
Salmonella ZFL022P	+	Poultry
Salmonella ZFL024P	+	Poultry

Key: **+** = β-lactamase Present; **-** = β-lactamase Absent., NIMR= Nigeria Institute of Medical Research, ZFL=Zartech Farms Laboratory.

Conclusion

It is recommended that there be a surveillance of emerging resistant *Salmonella* pathogens not only in clinical settings but also in healthy individuals and environment. Previous studies conducted in Nigeria have demonstrated that *Salmonella* resistant gene reservoirs are increasing in healthy individuals (Okeke et al., 2000). The amazing build up of resistance in *Salmonella* species, especially amongst the poultry isolates as observed in this study has been encouraged by the indiscipline and complacency of most individuals in the developing countries (Abioye, 2002). This ranges from practices such as, poor sanitation to indiscriminate use of antimicrobial agents and subtherapeutic doses of antibiotics used.

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