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Antimicrobial Susceptibility and Occurrence of Resistance genes among *Salmonella* arizonae isolated from Chicken Meat samples in Sokoto Metropolis, Sokoto State, Nigeria

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Abstract
This study was conducted to determine the antimicrobial susceptibility and occurrence of resistance genes among *Salmonella* Arizonae isolated from chicken meat samples collected in Sokoto metropolis, Sokoto State Nigeria. A total of 400 Chicken's Meat samples (breast muscle) were collected and examined for the presence of *Salmonella* organisms. The isolated *Salmonella* Arizonae were then tested for antimicrobial susceptibility using disc diffusion technique and antimicrobial resistant isolates were then examined for the presence of ampicillin resistance gene *bla*TEM, chloramphenicol resistance gene *flo*R and tetracycline resistance gene *tet*A by PCR. *Salmonella aizonae* were isolated in only 4.5% (18 / 400) of the samples examined. Antimicrobial susceptibility test of the isolates showed susceptibility to nitrofurantoin (94.4%), nalidixic acd (94.4%), oflxacin (77.8%), augmentin (72.2%) and cotrimoxazole (66.7%). However, the isolates were resistant to tetracycline (88.9%), ampicillin (72.2%) and chloramphenicol (66.6%). Only 8 of these isolates were resistant to one or more of the antimicrobial agents tested. Resistance gene analysis of the resistant isolates shows the presence of ampicillin resistance gene, *bla*TEM in 75% (6 / 8) of the tested isolates. Presence of chloramphenicol resistance gene, *flo*R was also detected in 37.5% (3 / 8) of the isolates. Presence of multiple resistance genes was also detected in the isolates. A combination of two different resistance genes was detected in 62.5% (5 / 8) of the isolates and presence of all the three resistance genes (*bla*TEM, *tet*A and *flo*R) was detected in one of the isolates. Antibiotics have been used widely in poultry to treat, prevent infections and also in feeds to promote growth. Such practice has improved poultry performance effectively and economically but has resulted in the increase in the spread of resistant bacterial strains. In our study, a high resistance profile of 88.9% to tetracycline, a popularly use drug in poultry industry and the presence of multiple resistance genes in 62.5% of the isolates was also observed. There is therefore the need for more rational use of antibiotics in animal production and more prudent use in humans.
Keywords: *Salmonella arizonae*, antimicrobial susceptibility, resistance genes, Sokoto metropolis Sokoto State, Nigeria.

INTRODUCTION

Advances in poultry production practices and heightened nutritional awareness have all combined to make Poultry products a leading source of protein for much of the world. Thus the incidence of enteric pathogens such as *Salmonella, E. coli* and *Campylobacter* infections in poultry flocks and associated incidence of antibiotic residues contamination of poultry products are of considerable public health significance (Rakesh, *et al.*, 2014).

Enteric pathogens infections remain the leading sources of food-borne illness throughout the world. Pathogens such as *Salmonella, E. coli* and *Campylobacter* have received considerable media attention. Infections of domestic poultry with enteric pathogens are course for concern both for the poultry industry and for the society. An alarming trend is the rapid emergence of antimicrobial agent-resistant enteric pathogens all over the world (David and Ban, 2001).

Salmonellae are Gram-negative rod-shaped bacteria, causing a wide range of human and animal diseases, such as enteric fever, gastroenteritis, endocarditis and bacteraemia (Bennasar *et al*, 2003, Liu, *et al.*, 2014). The sources of infection in humans are most commonly from contaminated food, water, meat or milk contaminated by human or animal faeces. Several studies have shown that sources of *Salmonella* infection in the poultry include contaminated products, feeds and feed ingredients, human wastes, mouse and rat droppings among others (Jones, 1992; Hayashi and Yamazaki, 1996).

Although infections with non-typhoidal salmonellae usually cause self-limiting diarrheic illness, serious sequelae include meningitis, sepsis and death may occur especially among infants and elderly persons (Glaser *et al.,* 1994). Non-typhoidal *Salmonella* invasive infections are a public health concern for infants, young children and adults with HIV (Gordon, 2012). Non-typhoidal *Salmonella* serovars are a leading cause of food-borne bacterial diseases in humans throughout the world (Erol *et al.,* 2013).

In Nigeria as well as in other developing countries of Africa and southern Asia, *Salmonella* infection pose a threat to public health with an estimated incidence of 33 million cases every year (Ivonoff, 1995; Sood *et al*., 1999). It is common in developing countries where it affects 12.5 million persons each year. Although it is widely believed that typhoid fever is endemic in Nigeria, the burden of the disease has not been objectively ascertained (Obaro *et al*., 2015). However, in Nigeria between 10% and 50% prevalence have been reported (Uttah *et al*., 2013). Treatment of patients has been based on the use of first line antibiotics such as chloramphenicol and co-trimoxazole, and the third generation cephalosporins. However, efficacy of some of these drugs has been doubtful, following the emergence of multi-drug resistance in *Salmonella* strains (Akinyemi *et al.,* 2002; Smith *et al.,* 2005). The emergence and spread of *Salmonella* strains having multiple resistances to nearly all commonly used drugs is a major challenge to health care system, reducing effective treatment options, increasing treatment costs and increasing the risk of complications and death (Kariuki, 2008). Thus the high rate of hospitalisation and prolonged illness of patients with *Salmonella* associated diseases as a result of treatment failure with empirical therapy has been a great concern to the public health authorities. Antibiotics are used by the poultry industry and poultry Veterinarians to enhance growth, feed efficiency and reduce disease. Approximately 80% of all food-producing animals receive medication for part or most of their lives (Lee *et al.,* 2001). The use of antibiotics as prophylactics and growth promoters at concentrations lower than those used for treatment is a potentially dangerous practice that can encourage the production of antibiotic resistant bacterial strains (Al-Mustafa and Al-Ghamdi, 2000). The selective pressure created by wide spread use of antimicrobial drugs in animals and humans may have contributed to the dissemination of resistant bacterial strains. Resistance markers may be carried on plasmids or chromosomes of resistant bacteria. Plasmids have been a major factor in the spread of antibiotic resistance between bacteria. Under antibiotic selective pressure, R-plasmids spread resistance markers between both homologous and heterogeneous bacteria (Halawani and Shohayeb, 2008). The emergence and spread of antibiotic resistance is a global concern for both human and veterinary medicine (Usui *et al.,* 2014).

This study was conducted to determine the antibiotic susceptibility profile and antimicrobial resistance genes of *Salmonella* Arizonae isolated from Poultry (Chicken) meat samples collected in Sokoto metropolis, Sokoto State Nigeria.

MATERIALS AND METHODS

Four hundred chicken's breast muscle samples were randomly selected over a period of one year. Samples were transported in ice pack to the Laboratory of Faculty of Medical laboratory Science, Usmanu Danfodiyo University Sokoto for processing.

Isolation of Salmonella species from chickens' meat sample

Isolation of the organism was carried out in accordance with the guide lines of ISO 6573 (2002) manual of detection of Salmonella species from food and animal feeds. Briefly, twenty-five gram (25g) of chicken breast muscle sample was pre-enriched at 37°C in 225ml of buffered peptone water, BPW (Becton and Dickinson, USA) in a shaker incubator overnight. Zero point five ml (0.5ml) of the pre-enriched culture was transferred to 10ml of selenite-F broth and incubated at 37°C overnight. There after one loopful of broth was sub-cultured and streaked onto MacConkey's agar plate (Becton and Dickinson, U.S.A) and incubated at 37°C for 18 hours. Non-lactose fermenting organisms; colourless, semi-transparent or opaque colonies typical for *Salmonella* organisms on MacConkey agar were sub-cultured onto *Salmonella-Shigella* (SS) agar (Difco) plates. The plates were then incubated at 37°C for up to 72 hours. Presumptively identified typical *Salmonella* colonies that are colourless or very light pink, opaque or semi-transparent mostly with black centres (due to hydrogen sulphite production) on the agar plates, were sub-cultured and inoculated onto and nutrient agar plates for purification prior to biochemical characterization of the isolates.

Biochemical characterization of the isolates

Presumptively identified isolates were inoculated onto triple sugar iron (TSI) agar, lysine iron agar (LIA), Urea agar, Simon citrate agar slants and also inoculated into buffered glucose-peptone water for methyl-red Voges-

Proskauer test, maltose, mannitol sugar utilization and motility tests were also carried out as part of biochemical tests on the isolates. Isolates that were oxidase negative, indole negative, methyl-red test positive, that shows red slant/yellow butt(alkaline/acid reaction), with or without hydrogen sulphite production on TSI slants were further characterized using Micro-gen test kit (Microbact™ Gram-negative identification kit, Microgen Bioproduct Ltd, UK). Later, serological test with Serum "O" and "H" (Murex Diagnostics, Kent, UK) by precipitation reaction was carried out for more identification of the isolates.

Antibiotic susceptibility testing

Preparation of media and bacterial inoculum

The bacterial isolates were tested for antibiotic susceptibility using Kirby-Bauer technique (Elmer, 1992). Mueller-Hinton agar medium (OXOID, UK) was prepared in a uniform thickness in 90mm diameter Petri dishes. Five (5) well-isolated colonies of test organism were selected and inoculated into brain heart infusion broth. It was standardized to match 0.5 McFarland standard (corresponds to approximately 1.5 X 10^8 CFU/ml). The adjusted suspension was used as inocula for the susceptibility testing. Commercially prepared antibiotic disks (Oxoid, UK) containing; Ampicillin 10µg (Amp), Cotrimoxazole 25µg (cot), Gentamicin 10µg (gen), Nalidixic acid 30µg (nal), Chloramphenicol 30µg (chl), Tetracycline 30µg (tet), Ofloxacin 30µg (flo) and Nitrofurantoin 30µg (nit) were obtained and used for the susceptibility testing.

Susceptibility testing procedure

This was carried out based on the procedure in CLSI (2006).

A freshly prepared Mueller-Hinton agar was inoculated with the diluted test organisms using a swab and allowed to stand for 5 minutes to adsorb. The antibiotic disk was placed onto the surface of the inoculated media and incubated at 37°C for 18 hours. At the end of the incubation period the zone of growth inhibition surrounding the antibiotic disk was measured and interpreted using the break point of Basic Laboratory Procedures In Clinical Bacteriology (Vandepitte, *et al.,* 1991).

Resistance genes

Isolates resistant to Ampicillin, Tetracycline and Chloramphenicol were further evaluated for the respective markers using PCR. For ampicillin resistant strains *bla*TEM, tetracycline resistant strains, *tet* (A) and for chloramphenicol resistant strains *floR*, genes were sought for respectively using appropriate primers (Table 1).

Extraction of genomic DNA

Genomic DNA was extracted according to Murugkar *et al*. (2003).

Briefly, bacterial isolates were grown in Brain Heart Infusion (BHI; Oxoid, UK) broth over night at 37°C and then the bacterial cells were harvested by centrifugation. The harvested cells were suspended in 3.5 ml sterile distilled water and boiled at 100°C for 10 minutes. After boiling, the samples were cooled on ice and immediately tested for the presence of genes sought using PCR analysis.

PCR amplification

PCR amplifications were performed using a master-mix kit containing the Taq DNA polymerase, PCR buffer, dNTPs (dATP, dTTP, dGTP) and $MgCl_2$. A volume of 2µL of the DNA template solution was added to 23 µL of the reaction mixture containing PCR buffer, 250 nmol/L primer and 1 U Taq DNA polymerase. Amplification was carried out in a thermal cycler (Biometra, Göttingen, Germany) with a temperature programme consisting of the initial denaturation (1 min at 94◦C), 40 amplification cycles (1 min 15s at 55◦C, 3min at 72°C, 5min at 72◦C), and the final extension (5 min at 72◦C). A volume of 10 µL of the PCR product was analysed by electrophoresis in a 1.2% agarose gel stained with ethidium bromide. A 100bp ladder was used as molecular marker.

Table 1: Primer sequence and PCR programmes for amplification of tetracycline, ampicillin and chloramphenicol resistant genes

Gene	Primer	Nucleotide sequence	amplicon size	Annealing temp.°c	Reference
1. *tetA*	F	GCTACATCCTGCTTGCCTTC	210	55	Fonseca et al., (2006)
	R	CATAGATCGCCGTGAAGAGG			
2. *blaTEM*	F	CATTTCCGTGTCGCCCTTAT	793	55	Randall et al., (2004)
	R	TCCATAGTTGCCTGACTCCC			
3. *floR*	F	AACCCGCCCTCTGGATCAAGTCAA	548	60	Randall et al., (2004)
	R	CAAATCACGGGCCACGCTGTATC			

1. 40 cycles (94°C, 1m; 55°C, 1m 15s; 72°C, 3m; 72°C, 5m)
2. 25 cycles (94°C, 1m; 55°C, 1m; 72°C, 3m)
3. 35 cycles (94°C, 1m; 60°C, 1m; 72°C, 2m; 72°C, 7m)

RESULTS

Out of the four hundred (400) meat samples evaluated, 26 were positive for *Salmonella* isolates, presenting 6.5% prevalence. Distribution of *Salmonella* serovars isolated from the chicken meat samples shows that of the 26 *Salmonella species* isolated, 18 (69.2%) were identified as *S.Arizonae,* 5 (19.2%) as *S.* Pullorum and 3 (11.5%) as *S.* Gallinarum.

Salmonella arizonae isolates in the study shows 94.4% susceptibility to nitrofurantoin and nalidixic acid respectively, 77.8% susceptibility to ofloxacin and 72.2% to augmentin respectively and 66.7% susceptibility to cotrimoxazole. However resistance profile reveal the isolates to be 88.9% resistant to tetracycline, 72.7% to ampicillin and 66.6% resistant to chloramphenicol (table 2).

TABLE 2 Antibiotic susceptibility profile of *Salmonella* Arizonae isolated from Chicken meat samples in Sokoto metropolis

Antimicrobial agents	Susceptibility profile (n=18)		
	Susceptible (%)	Intermediate (%)	Resistant (%)
Cotrimoxazole	12 (66.7)	2 (11.1)	4 (22.2)
Ampicillin	4 (22.2)	1 (5.6)	13 (72.2)
Nitrofurantoin	17 (94.4)	0 (0.0)	1 (5.6)
Nalidixic acid	17 (94.4)	1 (5.6)	0 (0.0)
Oflxacin	14 (77.8)	3 (16.7)	1 (5.6)
Augmentin	13 (72.2.)	5 (27.8)	0 (0.0)
Chloramphenicol	6 (33.3)	0 (0.0)	12 (66.6)
Tetracycline	2 (11.1)	0 (0.0)	16 (88.9)

Ampicillin resistance gene (*bla*TEM), tetracycline resistance gene (*tet*A) and Chloramphenicol resistance gene (*flo*R) were all detected in the tested 8 *Salmonella arizonae* isolates (Plate 1). Ampicillin resistance gene (*bla*TEM) and tetracycline resistance gene (*tet*A) were detected in 6 (75%) of the isolates respectively. Presence of multiple resistance genes was also observed in this study. In 5 (62.5%) of the isolates presence of a combination of two different resistance genes were detected (*bla*TEM / *tet*A, *bla*TEM / *flo*R and *tet*R / f*lo*R resistance genes). One (12.5%) isolate was observed to harbor all the three (*blaTEM, tet*A *and flo*R) resistance genes tested in the study.

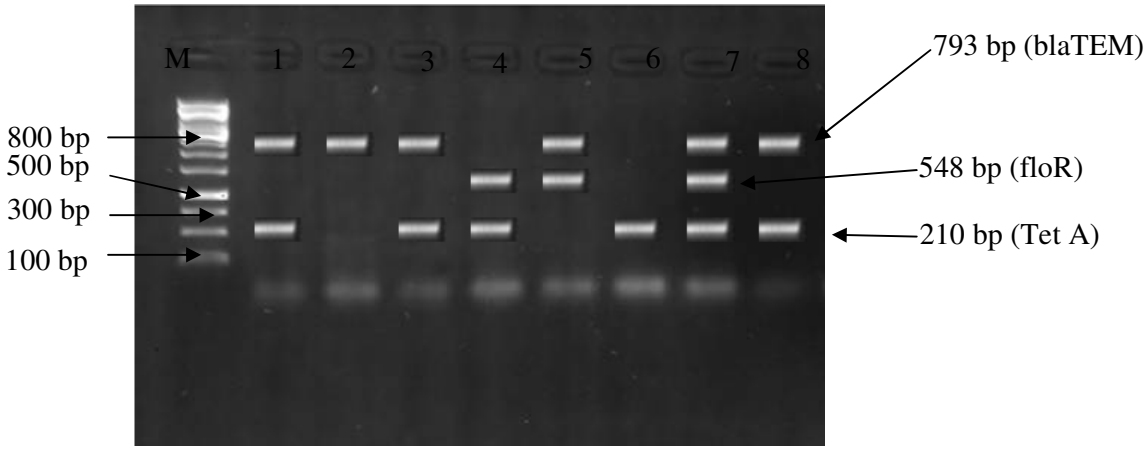

Plate 4.5: Agarose gel electrophoresis of *bla*TEM, *flo*R and *Tet*A genes after multiple-flex PCR test
M= Molecular weight maker/ DNA ladder
Lanes 1- 8 are the samples loaded with lines 1, 2, 3, 5, 7 and 8 showing amlicons for the blaTEM gene
Lanes 4, 5 and 7 showing amplicons for the FloR gene
Lanes 1, 3, 4, 6, 7 and 8 showing amplicons for Tet A gene

DISCUSSION

Antimicrobial resistance in *Salmonella* organisms is a cause of serious concern in both human and veterinary practice. In our study antibiotic susceptibility profile of the isolates showed 94.4% susceptibility to nitrofurantoin and nalidixic acid respectively, 77.8% susceptibility to ofloxacin, 72.2% susceptibility to augmentin and 66.7% susceptibility to cotrimoxazole. However, 88.9% of the isolates showed resistance to tetracycline, 72.2% of the isolates were resistant to ampicillin and 66.6% were resistant to chloramphenicol.

Varying tetracycline resistance profile among *Salmonella* species has been observed by many workers (Gordana, *et. al.,* 2012). Tetracycline resistance among *Salmonella* organisms seemed to vary according to the animal source and the geographical origin of the isolates (Frech and Schwarz, 2000). The result observed in this study is slightly higher than what was obtained by other researchers. Frech and Schwarz reported 92% tetracycline resistance in poultry. Tetracycline drugs are by far the most frequently used antimicrobial agents in veterinary medicine, ß-lactam antibiotics and aminoglycosides rank in third and fourth place. Resistance to aminoglycosides and ß-lactams was frequently encountered among tetracycline- resistant isolates (Frech and Schwarz, 2000, Toro *et al.,* 2011). Seventy-two point two percent (72.2%) of the isolates in this study were resistant to ampicillin the result is lower than what was reported by Agada *et al.* (2014) who reported a resistance of 96.0% to ampicillin in Jos, Nigeria. However, a higher (66.6%) chloramphenicol resistance was observed among the *Salmonella* Arizonae isolates in this study compared to 42.9% reported by Agada *et al.* (2014) in Jos, Nigeria. The observe increase in resistance to chloramphenicol in our study might be as a result of the continued miss-use and over-use of the drug by the public. Furthermore, wide spread use of antibiotics in medical, veterinary and agricultural practices have resulted in resistance to large spectrum of antibiotics leading to the proliferation of antibiotic resistance genes in the horizontal gene pool (Meervenne, *et al.,* 2012, Mubito *et al.*, 2014). The World wide increase in the use of antibiotics as an integral part of the poultry and livestock production industry has lead to the problem of the development of antibiotic resistant bacterial strains. Scientific evidence has shown that resistance to antibiotics is not only due to the natural ability of a tiny fraction of the bacteria with un-usual trait to survive antibiotic attack, enabling resistant strains to multiply but also stems from the transmissibility of acquired resistance to their progeny and across to other unrelated bacterial species through extra-chromosomal DNA. The circumstances of occurrence and spread of antibacterial drug resistance is complex, however, genetic analysis has indicated that the source of resistance is frequently a transferable plasmid (Lin, *et al.,* 2004; Abdullahi *et al.,* 2015). In this study, genotypic identification of *bla*TEM, floR and *tet*A genes were made among the 8 multi-drug resistant isolates. It was observed that among the multi-drug resistant isolates 5 (62.5%) of the isolates were found to harbor both *bla*TEM and *tet*A genes together, or blaTEM and floR or floR and tetA resistance genes combination. One (12.5%) isolate was found to harbor all the three resistance genes tested (*bla*TEM, *tet*A and *flo*R genes). This finding is in line with the findings of Huque *et al.*, (2001); Asma et al., (2005) and Abdullahi *et al.*, (2015), who reported the presence of multiple resistance genes in *Salmonella* isolates. Application of antibiotics in poultry production brings about an increase in resistance to antibiotics not only in pathogenic bacterial strains but also in commensal bacteria. In this respect, gastrointestinal commensal bacteria constitute a reservoir of resistance genes for pathogenic bacteria. Poultry and meat are common reservoirs of emerging antibiotic resistance available to bacteria inhabiting humans. It can be supposed that the transmission of antibiotic resistant bacteria to people who got in contact with these sources through direct ingestion or handling results in an increase in the human reservoir of these strains which can rapidly spread to the community. The emergence and spread of resistant bacterial strains like *Salmonella* from poultry products to consumers put humans at risk to new strains of bacteria that resist antibiotic treatment. Some of the significant implications of introducing antibiotic resistant bacteria to humans include increasing cost of treatment and suffering to individuals, families and the entire community. The emerging resistant bacterial strains will adversely affect the efficacy of antibiotic chemotherapy and will furthermore encourage the need for more expensive and toxic medication. The strategy to slow down the development of antibiotic resistant pathogens is to regulate antibiotic drug usage and also put in place resistance surveillance programs in the country.

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