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*Full Length Research Paper*

# Prevalence and antibiotic resistant salmonella in retail broiler chickens in Ibadan, Oyo State, Nigeria

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Poultry meat has been identified as one of the principal food-borne sources of salmonella. In this study, the prevalence of contamination with Salmonella spp. of broiler carcasses sold for human consumption at retail markets in Ibadan metropolis were identified and confirmed by cultural and molecular methods, susceptibility of the isolates to commonly used antibiotics was also determined. Two hundred samples were randomly collected from broiler carcasses from selected retail markets in Ibadan metropolis. The presence of salmonella spp. in the samples was determined after pre-enrichment, enrichment and culture on selective agar. Biochemical tests and molecular identification were further carried out to confirm the isolates. Polymerase Chain Reaction (PCR) assays was carried out using the invA specific primers for the detection of Salmonella spp. The confirmed isolates were then subjected to antibiotic sensitivity test using the disc diffusion method. Cultural isolation and biochemical characterization of retail broiler carcasses yielded 25.5% prevalence while molecular identification (PCR) yielded 4% prevalence. Antibiotic sensitivity test showed 100% resistance to Cefixime, Celflozidime and Oxytetracycline. Also, a high percentage resistance of Gentamicin (87.5%) and Cefuroxime (75%) to the isolated Salmonella spp. was observed. The prevalence of salmonella spp. from broiler chickens sold for consumption in the selected retail markets in Ibadan metropolis obtained using the molecular identification (PCR) method- 4% should be considered the true picture of the prevalence. Therefore, in order to provide a more accurate profile of the prevalence of Salmonella spp. in broiler carcasses, it is pertinent to use invA gene specific PCR method that could be considered as an appropriate alternative (Standard) to conventional culture method. The high percentage of resistance to the commonly used antibiotics obtained in this study could also be attributed to the irrational, persistent, unrestricted and uncontrolled use of these antibiotics in poultry production as curative; prophylactic or growth promoter in the study area. This study established the need to improve on hygiene at the point of slaughtering and dressing of broiler chickens so as to eradicate Salmonella spp. from poultry markets. Also, the need for proper use of antibiotics to increase bacteria sensitivity to antibiotic for better results when needed was emphasized.

**Keywords:** Salmonella, broilers, polymerase chain reaction (PCR), antibiotic sensitivity

## INTRODUCTION

Salmonella is one of the most common pathogens implicated in food-borne illness with estimated cause approximately 1·4 million illnesses annually in the USA (Mead *et al.*, 1999). The organism has been isolated from a range of foods in almost every country in which it has been investigated from foodstuffs such as meat from swine, poultry, poultry products and dairy products (Manie *et al.*, 1998; Cloak *et al.*, 1999; Duffy *et al.*, 1999; Gebreyes *et al.*, 2000; Rajashekara *et al.*, 2000). Broiler chicken serves as a source of poultry meat which has become a product accepted and consumed worldwide without cultural or religious obstacles associated with its use as food (Alabi and Alabi, 2009). Broiler meats obtained in Nigeria retail markets are either live birds from farms processed either on the farm or at the retail point and Chicken meat are readily available for sale in most retail markets throughout Ibadan.

During the slaughtering and dressing of chickens, there can be fecal contamination of the carcasses from the gut of the birds which means bacteria present in the spilled

gut content is passed on as contaminants. Salmonella is of an increasing public health concern because it is the most incriminated pathogenic microbe of bacterial food poisoning especially present in poultry meat with infection being through the handling of raw poultry carcasses and products. Prevalence of Salmonella in poultry meat using both traditional and conventional methods has been reported worldwide from retail outlets, retail markets and processing plants. In retail markets, prevalence was reported in broiler at 10.60% in Croatia market (Kozačinski *et al.***,** 2006), 35.50% in Mexico (Miranda *et al*., 2009) and 5.92% in Saudi Arabia (Moussa *et al*., 2010). In Nigeria, several rates have been reported with 11.10% prevalence in Calabar metropolis (Ukut *et al*., 2010) and 2.0% in Oshogbo (Adesiji *et al.,* 2011). Salmonella resistance to Ampicilin appears to be the most common in Nigeria, followed by Trimethoprim-sulphamethozazole, Streptomycin, Cephalexin, Gentamycin (Enabulele *et al*., 2010), and more than 90.0% resistance to Tetracycline (Sakaridis *et al*., 2011). The aim is to find the prevalence and antibiotic resistant Samonella in retail broiler chickens in Ibadan, Oyo state Nigeria.

## MATERIALS AND METHODS

### Collection of samples

The study was carried out in the core metropolis of Ibadan, Oyo State, Nigeria (because Ibadan is the hub of poultry production in Nigeria) which consists of five Local Government areas (LGAs), namely: Ibadan North, Ibadan North West, Ibadan North East, Ibadan South West and Ibadan South East. Five retail markets were randomly selected from the five Local Government Areas. Eight (8) retail points were randomly selected at each selected market and five (5) meat samples from different broiler chicken were collected from each retail point, making a total of forty (40) retail points and a total of two hundred (200) broiler chicken meat samples collected. The sample size was determined using the Thrusfield method, (2005) formula, with an expected prevalence of 11.1% according to Ukut *et al.* (2010), confidence interval of 1.96 and desired absolute precision of 5%. The samples were collected in sterile sample bottles, properly labeled, placed in ice pack and transported in cool boxes to the Food and Hygiene Laboratory of the Department of Veterinary Public Health and Preventive Medicine, University of Ibadan, Ibadan, Nigeria for bacteriological analysis.

### Laboratory procedure

The methods that were used were based on:
(i) Microbiological Standards and Guidelines by USDA (National Agricultural Library) (USDA, 2011).
(ii) NCCLS guideline from Global Salm-Surv, (2003).

Horizontal method for the detection of salmonella spp. From food and animal feeding stuffs (ISO Standard Catalogue 07.100.30: WHO, 2011).

### Bacteriological isolation of salmonella

### Pre-enrichment stage

25 g of broiler chicken meat was weighed and blended in a stomacher machine for 2 min, 1.5g of it was weighed out and homogenized in 13 ml of 20% buffered Peptone water (lab M, UK) in a bijou bottle which gave a dilution ratio of 1:10. These were incubated at 37°C for 24 h. 20% buffered Peptone water was prepared by dissolving 20 g peptone water powder into 1000 ml of distilled water. Sterilization was done by autoclaving it for 15 min at 121°C.

### Enrichment

Enrichment medium-Thiothionatebroth (TT broth) was prepared by suspending 89.5 g of Thiothionate in 1000 ml of distilled water it was boiled and then cooled just below 45°C immediately before use, 20 ml of Iodine-Iodine solution prepared by dissolving 25 g of potassium Iodide in 10ml of distilled water and 20 g of Iodine crystal was added and was diluted to 100 ml with sterile distilled water. The content of one vial of Novobiocin supplement (SRO181E) reconstituted as directed (2 ml of sterile distilled water) was added per 250 ml of the broth. These were well mixed and were aseptically dispensed into sterile bijou bottles. 1ml of the pre-enrichment broth was added to 10 ml of the prepared enrichment broth and these were incubated for 24 h at 37° ± 1°C.

### Agar plating

Plating of the incubated enrichment broth on selective growth medium (agar). The selective growth medium used was Salmonella/Shigella agar (SSA). SSA was prepared by weighing 63.2 g of the agar, mixed with 1000 ml of sterile distilled water then boiled (no autoclaving) molting agar was poured into sterile petri dishes and were allowed to cool , gelled and solidified. 10 µl of the enrichment broth was poured onto dishes and streaked on the agar. These were then incubated at 37° ± 1°C for 24 h.

### Sub-culturing of presumptive positive plates

51 presumptive positive plates were sub cultured onto MacConkey agar and were incubated at 37° ± 1°C for 24 h.

MacConkey agar was prepared by weighing 49.5 g of the agar, mixed into 1000 ml of distilled water. The solution was boiled and then sterilized by autoclaving at 121°C for 15 min. A wire loop of the presumptive positive plates were sub-cultured onto the prepared agar and then incubated for 24 h at 37° ± 1°C.

**Morphological identification of the isolated organism**

The presumptive positive samples on Salmonella-Shigella (SS) Agar produced lactose non-fermenting colonies with black centers while on MacConkey Agar produced non lactose fermenting smooth colonies, that is, pale colonies.

**Biochemical characterization of isolation**

**Catalase test**

Clean glass slides were placed horizontally on the work bench, a drop of 5% hydrogen peroxide ($H_2O_2$) was placed on the slides corresponding to the number of the presumptive positive samples. A clean and sterile wire loop was used to transfer a small quantity of the presumptive positive colony on to the drops of the $H_2O_2$.

**Urea test**

24.5 g of urea agar was dissolved in 950 ml of sterile distilled water; 5 ml of 40% urea water was added and sterilized by autoclaving for 15 min at 121°C. This was made into slant in sterile bijou bottle and allowed to cool, gel and solidified. A loopful of the isolate was streaked on the slant before it was incubated for 24 h at 37° ± 1°C.

**Indole test**

13.0 g of Nutrient broth was dissolved in 1000 ml of sterile distilled water and sterilized by autoclaving at 121°C for 15 min, 5 ml of this was dispensed into sterile test tubes, this was inoculated with isolate sterilely sealed and incubated for 24 hours at 37°C ± 1°C. 0.5 ml of Kovac`s reagent was added to the broth culture, the presence or absence of colored ring was noted.

**Triple sugar iron (TSI) test**

52.5 g of TSI agar was dissolved in 1000 ml of sterile distilled water, autoclaved for 15 min at 121°C. Slants were made from the agar where the presumptive positive colonies were inoculated with a sterile inoculating wire. These were incubated for 24 h at 37° ± 1°C.

**Molecular identification**

**Extraction of DNA template for polymerase chain reaction (PCR)**

**Method**

Salmonella isolates from the cultured and biochemical tests were sub cultured onto nutrient agar and incubated for 24 h at 37°C. Presumptive positive isolates from each plates were scooped and dissolved in 5ml of sterile distilled water, 1 ml of it was dispensed aseptically into a Eppendorf tube, This was heat till boiling in a water bath at 100°C for 10 min this was cool on ice and vortexed into sterile Eppendorf tube and was centrifuged at 6000 rotations per minute for 5 min. The supernatants were used for amplification by PCR with salmonella specific primers.

**Primers set and PCR amplification program**

Salmonella specific primers (Rahn *et al.,*1992) have respectively the following nucleotide sequence based on the invA gene of Salmonella primers for specific detection of Salmonella at :GTG AAA TTA TCG CCA CGT TCG GGC AA - 3´and 5´ TCATCG CAC CGT CAAAGG AAC C-3´, forward and reverse primer. Reactions with these primers ware carried out in a 50 µl amplification mixture consisting of 25 µl of PCR Master Mix (Genei, Bangalore), 2 µl of each primer, 19 µl grade water and 2 µl isolate were used in the reaction. This total of 50 µl mixture in the Eppendorf tubes was put in the thermocycler which heat and cool the reaction tubes to achieve the temperature required at each step.

**PCR action**

Amplification was conducted in Master-gradient Thermocycler (Eppendorf). The cycle conditions were as follow:

An initial incubation at 94°C for 60 sec Followed by 35 cycles of denaturation at 94°C for 60 sec, annealing at 64°C for 30 sec and elongation at 72°C for 30 sec, followed by 7 min final extension period at 72°C.

**Agarose electrophoresis**

Agarose gel electrophoresis was employed for size separation of the PCR products. The size of the PCR product was determined by comparing with a DNA ladder (a molecular weight marker), which contains DNA fragments of known size (100 base pair) run on the agarose gel alongside the PCR products.

A known salmonella strain (Salmonella typhi ATCC 14028) used as reference stain was used as positive control and distilled water used as negative control.

## Antibiotic sensitivity test

Disk diffusion antibiotic sensitivity test was used in determining isolates that were sensitive to the following commonly used antibiotics at the stated micrograms (using the Kirby- Bauer – (K- B) disc diffusion antibiotic testing method).

CAZ - Ceflazidime - 30µg, CRX - Cefuroxime - 30µg, GEN - Gentamicin - 10µg
CXM - Cefixime - 5µg, OFL - Ofloxacin - 5µg, AUG - Augumentin - 30µg
NIT - Nitrofurantion - 300µg, CPR - Ciprofloxaxin - 5µg, TET - Oxytetracycline - 30µg

Mueller Hinton agar was prepared by dissolving 38 g of the agar into 1000 ml of sterile distilled water; this was autoclaved for 15 min at 121°C. This was plated, allowed to cool, gelled and solidified. Multiple antibiotic sensitivity disks was used (100 sensitivity rings NZ53N-Abteck Biological Limited) by impregnating the agar with the antibiotic sensitivity disk before it was incubated at 37° ± 1°C for 24 h.

## Statistical analysis

Data were presented in tables, percentages and graphs with Excel spread sheet.CHI square was used to compare prevalence of Salmonella spp. in the 5 different broiler retail markets – Mokola (1), Aleshinloye (2), Molete (3), Apata (4), and Bodija (5) markets. The standard deviation was also calculated.

# RESULTS

## Prevalence of salmonella species

### Cultural isolation

The results obtained from the cultural isolation and biochemical tests showed that 51 broiler chicken meat samples out of 200 samples were contaminated with Salmonella spp. comprising 40%, 30%, 27.5%, 17.5% and 12.5% from samples Apata, Molete, Bodija, Mokola and Aleshinloye markets respectively as shown in (Table 1).

### Biochemical test

Results showed that 51 samples were positive viz; 15 were catalase positive, 11 were urea test positive, 10 were indole test positive and 15 were triple sugar iron test positive as shown in (Table 2).

## Polymerase chain reaction

DNA genomes extracted were used in the PCR. The 51 presumptive positive isolates from cultural method were subjected to molecular identification. Only 8 were confirmed to be salmonella spp. positive as shown in (Figure 1 and Table 3).

## Antibiotic sensitivity test result

Antibiotic sensitivity test was conducted on the eight isolates confirmed via PCR. Three of the isolates were sensitive to Augmentin, four to Ofloxacin, and one to Gentamicin. Whereas, six of the isolates were sensitive to Ciprofloxacin, seven to Nitrofurantoin, two to Cefuroxime while none of the isolates was sensitive to Cefixime, Celflozidime and Oxytetracycline (Table 5 and Figure 2).

# DISCUSSION

Salmonella spp. was isolated through culture in only 51 meat samples out of 200 that were selected for this study, yielding a prevalence of 25.5%. Prevalence of Salmonella spp. was highest in broilers from Apata (40.0%) followed by those from Molete (30.0%), Bodija (27.5%), Mokola (17.5%) and Aleshinloye (12.5%). The difference in prevalence of Salmonella spp. by market type was statistically significant ($\chi^2 = 9.84$, $p = 0.04$).

The prevalence of Salmonella by molecular identification method (PCR) showed that the 4% obtained in this study is similar to findings of Salmonella spp. (0 - 5%) in retail market broiler chicken samples reported from different countries such as 2% in Osogbo, Nigeria, (Adesiji *et al.,* 2011), and 5.92% in Saudi Arabia (Moussa *et al.,* 2010), but still fall below some prevalence reported in Croatia market -10.60% (Kozačinski *et al.,* 2006 ); 35.50% in Mexico (Miranda *et al.,* 2009) and 11.2% in Calabar Metropolis (Ukut *et al.,* 2010). The close prevalence obtained within the different sample locations (broiler chicken retail markets) - mokola 2%; Aleshiloye 2%; Bode 1%; Apata 2% and Bodija 1% may be due to improved hygiene and processing methods since salmonella contamination of carcass is usually from the feacal contamination from the gut of the broiler chicken, which means bacteria present in the spilled gut content is passed on as contaminants on to the carcass or on the knifes used during slaughtering and dressing.

Antibiotic Sensitivity of Salmonella spp. from the study shows that three of the isolates were sensitive to Augmentin, four to Ofloxacin, and one to Gentamicin.

**Table 1.** Prevalence of salmonella spp. in broilers from Ibadan (Cultural method).

| Variable | No positive (%) | No sampled | $\chi^2$ | *p-value* |
|---|---|---|---|---|
| Market | | | | |
| Mokola | 7(17.5) | 40 | | |
| Aleshinloye | 5(12.5) | 40 | | |
| Molete | 12(30.0) | 40 | | |
| Apata | 16(40.0) | 40 | | |
| Bodija | 11(27.5) | 40 | 9.84 | 0.04 |

**Table 2.** Results of Biochemical characterization.

| Market | Catalase test | Urea test | Indole test | TSI test |
|---|---|---|---|---|
| Mokola( 1 ) | 3 | 2 | 3 | 3 |
| Aleshinloye (2) | 4 | 2 | 4 | 3 |
| Bode (3) | 3 | 2 | 4 | 3 |
| Apata(4) | 3 | 2 | 2 | 3 |
| Bodija (5) | 2 | 2 | 3 | 3 |

**Table 3**. Specimen used for PCR Agarose Gel Electrophoresis.

| DNA Ladder (gene molecular weight maker -100 base unit) | | | |
|---|---|---|---|
| A | | | |
| B | Specimen 1A | I | Specimen 5B |
| C | Specimen 1B | J | Salmonella reference strain (ATCC 14028 ) Salmonella typhi |
| D | Specimen 2A | K | Salmonella reference strain (ATCC 14028) Salmonella typhi |
| E | Specimen 2C | L | Specimen 6A |
| F | Specimen 3A | M | Specimen 3D. |
| G | Specimen 4A | N | Specimen 5E |
| H | Specimen 4C | O | Specimen 2D. |
| | | P | Distilled water as negative control |

**Table 4.** Diagnostic efficacy of the isolation method using PCR as the gold standard.

| Isolation | PCR | | Total |
|---|---|---|---|
| | Positive | Negative | |
| Positive | 5 | 46 | 51 |
| Negative | 3 | 146 | 149 |
| Total | 8 | 192 | 200 |

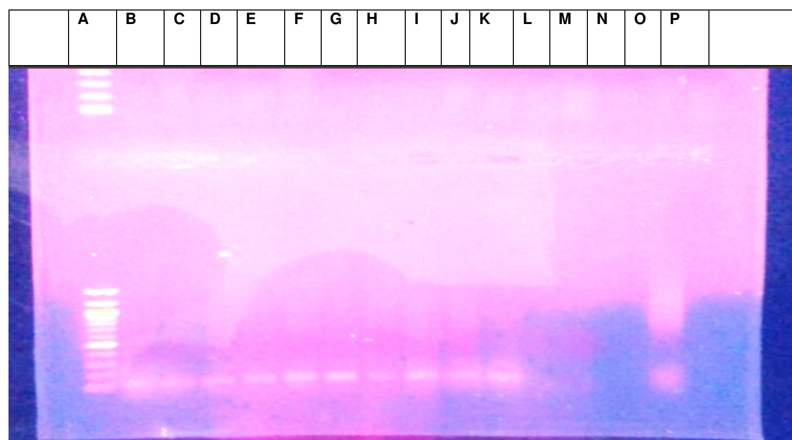


**Figure 1.** PCR Agarose Gel Electrophoresis results.

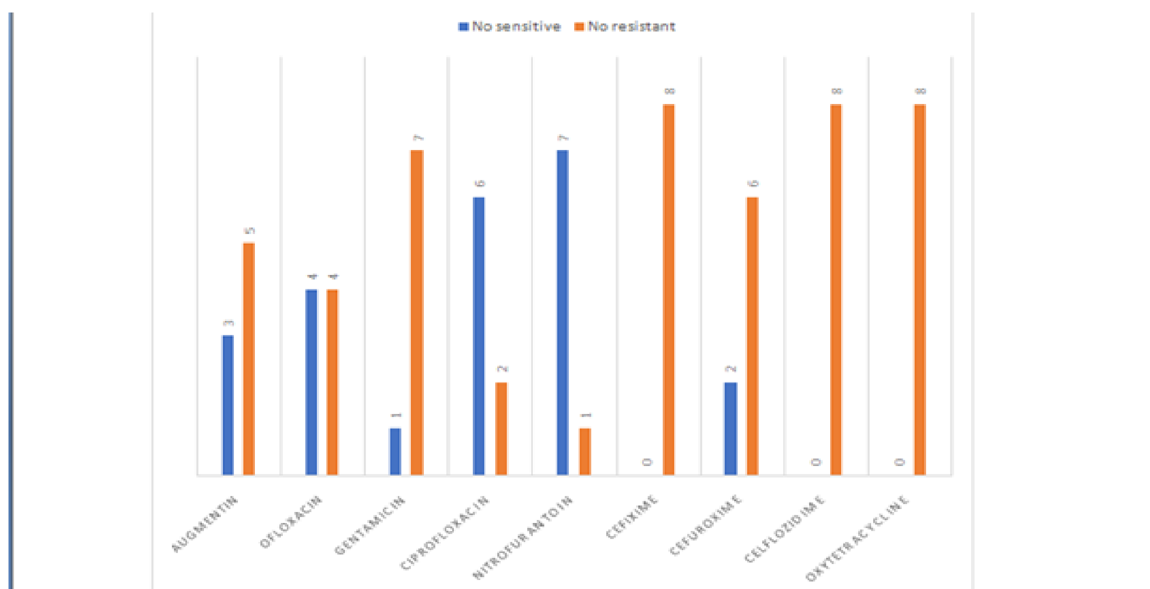


**Figure 2**. Graphical representation of results from antibiotic sensitivity test.

Moreover six of the isolates were sensitive to Ciprofloxacin, seven to Nitrofurantoin, two to Cefuroxime, while none of the isolates was sensitive to Cefixime, Celflozidime and Oxytetracycline. The 100% resistance of Cefixime; Celflozidime and Oxytetracycline coupled with very high percentage resistance of Gentamicin (87.5%) and Cefuroxime (75%) to the isolated Salmonella spp. could be attributed to the irrational, persistent, unrestricted and uncontrolled use of these antibiotics in broiler chicken production as either cure or prophylaxis.

The presence of multiple drug-resistant bacteria in the food chain has been reported by a number of investigators (D'Aoust *et al.,* 1992; Manie *et al.,* 1998; Duffy *et al.,* 1999; Gebreyes *et al.,* 2000; Rajashekara *et al.,* 2000), and more than 90.0% resistance to Tetracycline (Sakaridis *et al.,* 2011), a drug commonly used in poultry production in Nigeria raising concern for the medical consequences associated with food-borne illness.

## Conclusion

The result of this study indicated low incidence - prevalence of 4%. However, the presence of Salmonella in food for human consumption should not be encouraged as small number of contaminated carcasses may have an impact in spreading contamination. This study supported the ability of invA specific primer sets to confirm the isolates as Salmonella. There was a very high percentage of resistance to most antibiotics commonly used in broiler chicken production. The development of multi-drug resistance in the serotypes of salmonella has a significant impact on the antibiotic treatment of Salmonella infections and the lack of an effective antibiotic therapy may lead to an increase in the morbidity and mortality rates.

## Authors' declaration

We declared that this study is an original research by our research team and we agree to publish it in the journal.

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