Research Article

Detection, Serotyping and Antimicrobial Resistance Pattern of Salmonella from Chicken slaughter slabs in Jos, North-Central Nigeria

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Abstract | Poultry processing activities require large amount of high quality water for processing, cleaning and cooling. Contamination of water at any point in the processing stage is likely to affect meat hygiene. Waste water generated during these activities typically has high presence of organic material such as blood, fat, flesh and excreta. In developing countries like Nigeria where access to portable water is lacking, it is not uncommon to "reuse" rinse water for processing several batches of poultry carcass.Several samples were collected from three poultry slaughterhouses within Jos metropolis at intervals for a period of 6 months to check for presence of *Salmonella* during various stages of processing. *Salmonella* was isolated from at least one of the four matrices in the three LBM with an overall prevalence of 20.2%. Of the 84 samples collected, 17 contained *Salmonella*. With regard to the matrices, there was no difference in *Salmonella* prevalence among the four matrices considered. Four different serotypes were identified with *Salmonella* Llandoff having the highest isolation rate in all the matrices sampled (58.8%), followed by, *S.* Kentucky (17.7%), *S.* Schwarzengrund and *S.* Havana had the lowest isolation rate (11.8%). *S.* Llandoff was isolated in all the matrices and was distributed across the three LBM whereas the other less frequent serovars had a more circumscribed distribution. Resistance to Methicillin, Penicillin, Erythromycin and Oxacillin was 100% for all the serotypes while Gentamicin had the lowest resistance (5.9%).This is one of few studies on the occurrence and antimicrobial resistance profile of *Salmonella* in poultry slaughter slab (processing plants) in Nigeria. The relatively high prevalence rate documented in this study may be attributed to the generally poor infrastructure, lack of well-equipped poultry slaughter houses, lack or inadequate water supply at these markets which hampers the ability of handlers to maintain good sanitary and hygiene conditions of the carcass, environment and themselves. Data collected could be valuable for instituting effective intervention strategies for Salmonella control in Nigeria with the aim of reducing Salmonella spread from poultry to humans.

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Introduction

Salmonella infections in poultry are an important source of clinical and food borne disease of both animals and humans. Host-adapted poultry *Salmonellae* (*Salmonella* Pullorum and *Salmonella* Gallinarum) are responsible for severe systemic diseases in developing countries relative to countries with testing and eradication programmes (Pomeroy and Nagaraja, 1999; Snoeyenbos, 1991). Numerous serotypes of non-host adapted paratyphoid *Salmonellae* are often carried sub-clinically by poultry birds and thereby contaminating poultry and poultry products (Malorny et. al., 2003). Salmonellosis is one of the most common causes of food borne diarrheal disease worldwide and remains a major public health problem in many parts of the world (Riyaz-Ul-Hassan et al., 2004). Majority of the over 2650 recognized serotypes of *Salmonella*

infect both human and animals worldwide with signs ranging from fever, abdominal cramps, vomiting, diarrhoea and death (Guibourdenche et al., 2010; Scallan et al., 2011; Issenhuth-Jeanjean et al., 2014).

The importance of food as a vehicle for the transmission of salmonellosis has been well documented (Matsuoka et al., 2004; Mullner et al., 2009; Dallal et al., 2010). A variety of foods have been implicated as vehicles transmitting salmonellosis to humans, including poultry, beef, pork, eggs, milk, cheese, fish, shellfish, fresh fruit and juice, and vegetables (Espi et al., 2005; Mazurek et al., 2004; Varma et al., 2005; Acha and Szyfres, 2001). However, poultry, egg, meat and dairy products continue to be the most common food vehicles of *Salmonella* infection (Rodrigue et al., 1990; D'Aoust, 1994; Llewellyn et al., 1998). Bacterial organisms generally are a major safety concern to the meat industry where contamination can occur at multiple stage along the food processing chain either at the pre-harvest, harvest or post-harvest stages. Again, food handlers may re-contaminate a thoroughly processed or cooked meat meant for consumption (Oscar, 2013; Wagner, 2013). This is especially common in most developing countries where dressed carcasses are transported openly or kept in contaminated containers.

Despite the occurrence of *Salmonella* in raw and cooked poultry and poultry products as well as in beef meat in Nigeria (Mbata, 2005; Smith et al., 2012; Tafida et al., 2013), very little information is available on the occurrence of *Salmonella* at poultry slaughter plants/houses even when it has been shown to be the main source of cross-contamination in many parts of the world (Wang et al., 2013). This is even compounded by the fact that very few of those slaughter plants/houses exist and consumers are forced to sometimes purchase live birds from them for slaughter. These birds are processed in an open environment at a slaughter slab in the market with poor sanitary and hygienic conditions. It is not uncommon to see faecal contamination of carcasses from the gut during slaughter and processing of these birds. This may lead to contamination of the processing line, equipment and subsequently cross-contamination of non-infected birds and humans (Olsen et al., 2003; Rostagno et al., 2006).

This study was designed to determine the occurrence of *Salmonella species* in water used for de-feathering (Hot water) and washing/rinsing (carcass-rinse) as well as in the intestinal contents of the birds and processing contact-surfaces (table swabs) used in processing poultry carcasses at three poultry slaughter slabs in Jos. We also try to identify the antimicrobial resistant pattern of the isolates from these different sources.

Materials and Methods

Study area

The study was carried out at three poultry slaughter slabs in Jos, Plateau State, North Central Nigeria. The city has a population of over 900,000 and a tropical climate with near temperate temperature in the northern part of the state. It lies within the guinea savannah vegetation zones of Nigeria with an average annual rainfall of 1200mm. The weather and rocky terrain are very conducive for livestock and arable crop production.

Sampling procedure

Sampling was carried out at intervals using a convenience sampling method from three live bird markets in Jos Metropolis: Terminus and Chobe Markets in Jos-North local government area (LGA), and Bukuru market in Jos-South LGA.

Eighty-four (84) samples were screened for *Salmonella* from four different matrices using convenience sampling and on the willingness of butchers to collaborate. Sampling was carried out three times at two months intervals. These include 21 de-feathering (Hot) water and 21 washing/rinsing water (Carcass-rinse), 21 Processing contact-surfaces (table swabs) and 21 intestinal samples (Figure 1). All samples were collected after the de-feathering, carcass-rinse and contact of birds with table. Both environmental and water temperatures (de-feathering and carcass-rinse) were read using a thermometer. Samples were collected into sterile universal bottles, labelled, placed on ice and immediately transported to the laboratory for analysis.

Isolation and identification of *Salmonellae*

A modification of the *Salmonella* culture method ISO 6579: 2002 (Annex D) was used. Briefly, samples were pre-enriched in buffered peptone water in a 1:10 sample to broth ratio at 37^{0}C for 24hrs. 0.1ml of the pre-enriched broth was inoculated into 10ml of Rappaport-Vassiliadis (RV) broth for enrichment

and incubated for 24 hours at 37^{0}C. A loop full Cultures from RV was then plated simultaneously onto Brilliance *Salmonella* Agar (BSA) and Xylose Lysine Desoxycholate (XLD) medium and incubated at 37^{0}C for 24 hours (ISO, 2002). Suspected isolates were identified by the colour and morphology of the colonies on the agar plates.

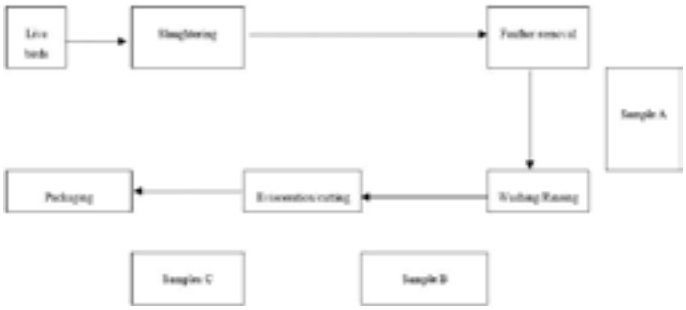

Figure 1: *The different stages of processing of chicken at a slaughter slabs in a live bird market*
Sample A: *Defeathering (Warm) water;* **Sample B:** *Washing/ Rinsing (Cold) water;* **Samples C:** *Intestinal contents and Processing surface (table swabs)*

Biochemical characterization of isolates

Biochemical test was carried out as described by (Raafat, et al., 2011). All isolates that gave the following reactions: indole negative, urease negative, citrate positive, motile in motility medium, oxidase negative, nitrate positive, lysine decarboxylase positive, Voges-Proskauer negative, ferment glucose, mannitol, dulcitol, and maltose but fail to ferment lactose, sucrose, inositol and raffinose were considered to belong to the genus *Salmonella.*

Evaluation of the resistant patterns of the isolates to antimicrobial agents

The resistant patterns of isolates to 10 antibiotics were determined using the agar-diffusion method (Khan et al., 2006). Five colonies were inoculated into a tube containing Tryptose soy broth (Difco, USA) and incubated overnight at 37^{0}C. Standardization of the inoculums was performed by diluting the broth cultures until turbidity matched the 0.5 McFarland standards. A sterile cotton swab was dipped into the standardized suspension, drained and used for inoculating 20 ml of Mueller-Hinton agar (Oxoid, UK) on a 100-mm disposable plate (Sterlin, UK). The inoculated plates were air dried, and antibiotic discs (Oxoid, UK) were placed on the agar using a disc dispenser. The following antibiotics were used: Sulphamethoxazole/ Trimethroprim (30 µg), Penicillin (10 µg), Chloramphenicol (30 µg), Gentamicin (10 µg), Erythromycin (5µg), Streptomycin (10 µg), Methicillin (10 µg), Oxacillin (5 µg), Cloxacillin (5 µg) and Nalidixic acid (30 µg). The plates were incubated aerobically at 37^{0}C for 18 to 24 h. The diameter of the zones of inhibition were measured and the sensitivity and resistance determined by the criteria of the Clinical and Laboratory Standards Institute (CLSI, 2006). *Escherichia coli* ATCC 25922 was used as the control strain. The formula: MAR index = (Number of antibiotics per isolate)/ (Total number of antibiotics tested) was used to calculate the multiple antibiotic resistance index (Wang et al., 2013, Adzitey et al., 2012) and all isolates that were classified as intermediate on the basis of inhibition zones were considered as sensitive for MAR index (Singh et al., 2010).

Detection of *InvA* gene using polymerase chain reaction (PCR) test

Three millilitres of each broth culture was centrifuged at 2,700 × g for 10 minutes to pellet the cells. The cells were washed twice with 1 ml PBS pH 7.4 and suspended in 200µl PBS pH 7.4. The genomic DNA was extracted using DNeasy® Blood and Tissue Kit (Qiagen, Netherlands) according to the manufacturer's instructions. The extracted DNA samples were stored at -20°C until used for PCR. Amplification of *invA* gene in *Salmonella* isolates was performed by using a primer pair specific to that locus as described by Rahn et al. (1992). The PCR was carried out in a 25µl comprising 5µl of the extracted DNA, 3.0µl of 10 × buffer (Fermentas®), 1.0µl of 10 mM dNTPs mix (Fermentas®), 1.0µl of 25 mM MgCl2, 1.25µl of 10 µM primers, and 2.5U of Taq Polymerase (Fermentas®). PCR amplification was performed in a GeneAmp® 9700 PCR System (Applied Biosystems) with initial denaturation at 95°C for 2 minutes followed by 35 cycles of denaturation at 95°C for 30 seconds; annealing at 53°C for 30 seconds, extension at 72°C for 1 min and final extension at 72°C for 7 minutes.

Ten microlitres of the PCR product was electrophoresed in a 1.5% agarose gel stained using 5µl of 10 mg/ml ethidium bromide at 100 V for 50 minutes. A 100bp DNA marker (Fementas®) was used as molecular size marker. The resulting gel was examined under a U.V transilluminator and DNA amplifications documented using a Gel Documentation System (Synegene®).

Serotyping of *Salmonella* isolates

Suspected *Salmonella* isolates were freeze-dried and shipped to the OIE Reference laboratory for

Table 1b: *Isolation frequency of Salmonella from de-feathering water, carcass-rinse, intestine and processing surfaces at LBM in Jos, Plateau State, Nigeria*

Live bird Market	Temperatures (°C)			No. of samples analysed				No. of samples positive for Salmonella (%)				
	Env Temp	De-feathering water	Carcass-rinse	Intestinal contents	De-feathering water	Carcass-rinse	Processing surface	Intestinal contents	De-feathering water	Carcass-rinse	Processing surface	Total
Terminus	18-20	48-51	22-24	7	7	7	7	2 (2.4)	2 (2.4)	1 (1.2)	3 (3.6)	8(9.5%)
Chobe	17-20	50-52	22-24	7	7	7	7	1 (1.2)	2 (2.4)	1 (1.2)	0 (0)	4(4.8%)
Bukuru	20-22	54-63	23-25	7	7	7	7	2 (2.4)	0 (0)	2 (2.4)	1 (1.2)	5(6.0%)
Total				21	21	21	21	5 (6.0)	4 (4.8)	4 (4.8)	4 (4.8)	17 (20.2)

Table 2: *Percentage Salmonella serotypes resistant to different antibiotics*

Serotypes	Percentage of each serotype resistant to each antibiotic (%)										Overall (%)
	SXT	C	NA	S	MET[a]	P[b]	E[c]	OX[d]	OB	CN	
S. Llandoff	50 (5/10)	20 (2/10)	60 (6/10)	20 (2/10)	100 (10/10)	100 (10/10)	100 (10/10)	100 (10/10)	80 (8/10)	10 (1/10)	68 (68/100)
S. Schwarzengrund	0 (0/2)	0 (0/2)	50 (1/2)	0 (0/2)	100 (2/2)	100 (2/2)	100 (2/2)	100 (2/2)	100 (2/2)	0 (0/2)	55 (11/20)
S. Havana	0 (0/2)	0 (0/2)	0 (0/2)	0 (0/2)	100 (2/2)	100 (2/2)	100 (2/2)	100 (2/2)	100 (2/2)	0 (0/2)	50 (10/20)
S. Kentucky	33.3 (1/3)	0 (0/3)	33.3 (1/3)	0 (0/3)	100 (3/3)	100 (3/3)	100 (3/3)	100 (3/3)	100 (3/3)	0 (0/3)	56.7 (17/30)
Overall	35.3 (6/17)	11.8 (2/17)	47.1 (8/17)	11.8 (2/17)	100 (17/17)	100 (17/17)	100 (17/17)	100 (17/17)	88.2 (15/17)	05.9 (1/17)	62.4 (106/170)

SXT: *Sulphamethoxazole/Trimethroprim;* **C:** *Chloramphenicol;* **NA:** *Nalidixic acid;* **S:** *Streptomycin;* **MET:** *Methicillin;* **P:** *Penicillin;* **E:** *Erythromycin;* **OX:** *Oxacillin;* **OB:** *Cloxacillin;* **CN:** *Gentamicin;* [a]**:** *All the isolates are resistant to Methicillin;* [b]**:** *All the isolates are resistant to Penicillin;* [c]**:** *All the isolates are resistant to Erythromycin;* [d]**:** *All the isolates are resistant to Oxacillin*

Salmonellosis (Istituto Zooprofilattico Sperimentale delle Venezie, Padova, Italy) for serotyping.

Results and Discussion

Determination of the occurrence of *Salmonella*

Five (23.8%) out of the 21 intestinal samples, 4(19.1%) out of the 21 table swab, 4 (19.1%) out of 21 de-feathering water sample and 4 (19.1%) out of 21 carcass-rinse sample yielded *Salmonella*. The overall isolation frequency of *Salmonella* was 17 (20.2%) out of the 84 samples tested (Table 1b).

Identification and characterization of isolates

All the eighteen (18) suspect isolates identified by the conventional biochemical test where further confirmed by polymerase chain reaction (PCR) and all except one suspected isolates tested positive for the genus *Salmonella.*

Antimicrobial resistance of *Salmonella* isolates

Sixteen (94.1%) of the 17 isolates were susceptible to Gentamicin, 15 (88.2%) to Chloramphenicol and Streptomycin, 11(64.7%) to Sulphamethoxazole/Trimethroprim, 9(52.9%) to Nalidixic acid and 2(11.8%) to cloxacillin. All (100%) isolates were resistant to Oxacillin, Erythromycin, Penicillin and Methicillin (Table 2). The resistance pattern shows that all the serotypes are resistant to at least four (4) antibiotics with *S.* Llandoff from hot water being resistant to all the antibiotics (Table 3).

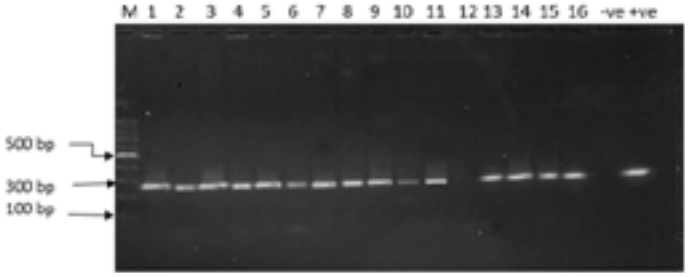

Figure 2: *Agarose gel electrophoresis of amplified invA gene of 16 Salmonella isolates. Lane 1-16 represents Salmonella isolates; -ve represent negative control; +ve represent positive control and M represent the molecular weight marker, 100 bp ladder*

PCR detection of isolates

All the 18 *Salmonella* suspects screened for *invA* gene detection using PCR gave a 284bp DNA fragment with exception of lane 11 which confirms that the isolates were *Salmonella* (Figure 2).

Salmonella Serotyping

The seventeen isolates serotyped were identified as *Salmonella* Llandoff (10 isolates), *S.* Kentucky (3 isolates), *S.* Schwarzengrund (2 isolates) and *S.* Havana (2 isolates).

Table 1a: *PCR primers used*

Target gene	Primer	Primer sequence	Size (bp)
invA	*invA*F	GTGAAATTATCGCCACGTTCGGGCAA	284
	*invA*R	TCATCGCACCGTCAAAGGAACC	

There are very few poultry slaughtering facilities in Nigeria and therefore live-birds especially the local chicken from different sources are sold and processed at the live-bird markets. On the other hand, some people patronize these live-bird markets because of cultural, religious or culinary reasons. In live-bird markets, consumers buy and take it to separate handlers for slaughtering at an open environment (slaughtering slabs). Consumers wait for their birds to be processed and delivered to them immediately thus making inspection of such processed carcasses difficult. In the United States, these LBM are exempted from Food safety and inspection service pathogen reduction performance standards (USDA, 2013). There is however, increased concern that such markets could be heavily contaminated with *S. enterica* (Imanishi, et al., 2014).

The ambient temperature, blood and moisture contents in these samples favour the multiplication and thriving of bacterial organisms. Occurrence of *Salmonella* in de-feathering (hot) water 4(19.1%) proves that *Salmonella* has become a big challenge in the processing of poultry meat especially in developing countries like Nigeria. *Salmonella* which was believed to grow at temperature of between 37^{0}C-42^{0}C was isolated in this study from de-feathering water with temperature between 48^{0}C-52^{0}C. Heat-processing should be sufficient to destroy any *Salmonellae* or reduce its incidence in poultry carcasses to an acceptable level, but may fail to do so, especially when temperature is inadequate or opportunities exist for recontamination of the finished product. From this study, it appears *Salmonella* may have become "acclimatized" to its environment and therefore, can survive or adapt to these temperature ranges. We were however not able to isolate *Salmonella* from de-feathering (hot) water at Bukuru market (Table 1a) probably due to the high temperatures of the water which might not be favourable for the survival of the organism. *Salmonella* was isolated from carcass-rinse 5(23.8%) used for washing/rinsing carcasses after de-feathering chicken. This is in agreement with Adeyanju and Ishola (2014) who reported cross contamination of poultry meat within the processing chain due to physical contact. *Salmonella* in our study may be as a result of cross-contamination from hot water or spillage of the gut contents as reported by (Orji et al., 2005) in Awka, Nigeria. *Salmonella* isolated from processing surfaces was low (19.1%) as compared to a prevalence of 25% and 23.5% reported in China and Malaysia (Wang et al., 2013; Adzitey et al., 2012) respectively. Isolation of *Salmonella* from the processing-surfaces could be as a result of contaminated carcass-rinse or evisceration of the gut contents, since the table is hardly washed or kept clean after every butchering. Our finding was supported by (Orji et al., 2005) who isolated *Salmonella* (12.5%) from poultry droppings in Awka south east Nigeria and some of this birds eventually get to slaughter slabs. The highest *Salmonella* isolates (23.8%) was obtained from the intestinal contents and carcass-rinse. This was however expected for the intestinal content due to their high microbial content and physical contact. Interestingly, the high incidence in carcass-rinse raises a lot of public health concerns.

Four different serotypes of *Salmonella* were identified from the isolates. *S*. Llandoff predominated with 11(74.6%), followed by *S*. Kentucky 3 (16.7%), *S*. Schwarzengrund 2(11.1%) and *S*. Havana 2(11.1%). The serotypes identified in our study differ from the ones reported by (Orji et al., 2005) in Awka, Nigeria. The difference can be due to study area and design. For public health concern, *S*. Schwarzengrund isolated in this study was earlier reported by Imanishi et al. (2014) as the cause of outbreak and infant death in the USA in 2007. The high isolation rate recorded in Terminus LBM may not be unconnected to the fact that it is the most active (high slaughter rate per day) of the 3 LBM markets examined due to the population density and demand.

All (100%) the isolates were resistant to at least four antibiotics namely Methicillin, Penicillin, Erythromycin and Oxacillin. Our findings were similar to that observed by (Obi and Ike, 2015) in Nsukka, Nigeria from an intensively reared and backyard poultry suggesting the growing threat of antibiotics on farms to the detriment of public health. The mechanism for bacterial antimicrobial resistance varies (Bennett, 2008) and given that an isolate (*S*. Llandoff) from hot water was resistant to all the antibiotics tested

Table 3: *Multiple antibiotic resistant patterns of Salmonella isolates*

Serotypes	Live bird Market and source	No. of antibiotics	Multiple antibiotic resistance pattern	No. (%) of isolates	MAR index
S. Llandoff	Terminus, (Intestine #4) Terminus, (Warm water #2) Bukuru, (Intestine #5)	4	MET+P+E+OX	3 (16.7)	0.4
S. Schwarzengrund	Terminus, (Intestine #3)	5	MET+P+E+OX+OB	1 (5.6)	0.5
S. Llandoff	Chobe, (Warm water #5)	5	MET+P+E+OX+OB	1 (5.6)	0.5
S. Kentucky	Bukuru, (intestine #2) Bukuru, (Cold water #2)	5	MET+P+E+OX+OB	2 (11.1)	0.5
S. Havana	Terminus, (Table swab #4) Bukuru, (Table swab #2)	5	MET+P+E+OX+OB	2 (11.1)	0.5
S. Schwarzengrund	Terminus, (Cold water #2)	6	MET+P+E+OX+OB+NA	1 (5.6)	0.6
S. Llandoff	Terminus, (Table swab #1)	6	MET+P+E+OX+OB+NA	1 (5.6)	0.6
S. Llandoff	Terminus, (Table swab #2) Chobe, (Intestine #4) Chobe, (Warm water #3)	7	MET+P+E+OX+OB+NA+SXT	3 (16.7)	0.7
S. Kentucky	Chobe, (Cold water #4)	7	MET+P+E+OX+OB+NA+SXT	1 (5.6)	0.7
S. Llandoff	Bukuru, (Cold water #4)	9	MET+P+E+OX+OB+NA+SX-T+C+S	1 (5.6)	0.9
S. Llandoff[a]	Terminus, (Warm water #1)	10	MET+P+E+OX+OB+NA+SX-T+C+S+CN	1 (9.1)	1

SXT: *Sulphamethoxazole/Trimethroprim;* **C:** *Chloramphenicol;* **NA:** *Nalidixic acid;* **S:** *Streptomycin;* **MET:** *Methicillin;* **P:** *Penicillin;* **E:** *Erythromycin;* **OX:** *Oxacillin;* **OB:** *Cloxacillin;* **CN:** *Gentamicin;* **#:** *Sample number;* **[a]:** *Isolate resistant to all the antibiotics*

(Table 3) is worrisome. The multiple drug resistance of all the isolates raises serious public health concern because they could pose considerable health risk to both consumers and handlers of poultry meat product. This study shows six (6) different resistant patterns with MAR index of 1 for one of the isolate (Table 3). The resistance to these entire antibiotics which are used both in humans and animals calls for serious public health concern. The percentage antibiotic resistance ranges from 5.6% (Gentamicin) to 100% (Methicillin, Penicillin, Erythromycin and Oxacillin) while the overall percentage serotype resistance to the 10 antibiotics is 62.4% (Table 2). Two of the ten *S.* Llandoff showed multiple antibiotic resistance indexes of 0.9 and 1.0 respectively.

Generally, the occurrence of *Salmonella* as a contaminant of poultry slaughter slabs 17(20.2%) is higher than the 12.5% that was obtained from chicken droppings in Nsukka but similar to the contamination of 21.0% of chicken meats in the province of Vietnam (Tran et al., 2005). Similarly, Wang et al. (2013) in China also reported a 22.1% rate of contamination by *Salmonella* and 22.6% reported by Adeyanju and Ishola (2014) in poultry processing plant in Ibadan, Nigeria.

In conclusion, our study demonstrated a high occurrence of *Salmonella* from four different matrices at three live-bird markets. The identification of Four different serotypes (*S.* Llandoff, *S.* Kentucky, *S.* Schwarzengrund, *S.* Havana) from 17 isolates that showed 7 different antibiotic resistant patterns against 10 antibiotics with the MAR index ranging from 0.4 to 1.0 suggest that lack of appropriate control measure and use of drugs is a potential "superbug" in the making. Poor infrastructure, lack of well-equipped poultry slaughter houses, lack or inadequate water supply at these markets hamper the ability of handlers to maintain good sanitary and hygiene conditions of carcass, environment and themselves. However, the unhygienic practices carried out at the Live-bird markets should be checked by the regulatory bodies responsible for maintaining good sanitary and hygienic conditions.

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Authors contribution

The study was conceptualized by DMM, AIO and IOF. IOF, PIA, BJA, SSN collected and analysed the samples while OIF, PDL, AIO, DOE wrote the draft manuscript. The project was supervised by MM, and LPM supervise the study and all authors read and approved the final draft for submission.

Conflict of interest

Authors declare no conflict of interest.

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