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The occurrence of multidrug-resistant *Salmonella nagoya* and other serovars in healthy commercial layers in Ilorin, Nigeria

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ABSTRACT

The occurrence of drug-resistant non-typhoidal *Salmonella* (NTS) in poultry has serious economic implications for the poultry industry and has the potential to cause human Salmonellosis. This study, therefore, aimed to determine the circulating serovars of NTS and their antibiotic susceptibility patterns in poultry in Ilorin. This cross-sectional study was conducted from January to March 2015. A total of 420 samples (cloacal, n=140; fecal, n=140; feed, n=70, and water, n=70) were aseptically collected from live adult birds from 14 farms using a systematic random sampling technique. *Salmonella* was isolated using the ISO 6579 method. Antibiotic sensitivity testing of NTS serovars was performed using the Kirby Bauer disc – diffusion method and interpreted using the epidemiological cut-off (ECOFF) values. The prevalence of NTS in poultry was 7.4% (n=31). Feed samples were the most contaminated samples (42%, n=13/31). Faecal sample (32%, n=10/31), cloacal swabs (19.5%, n= 6/31), and water samples (6.5%, n=2/31) also contained NTS. There was a significant difference between NTS isolation rates between farms ($p<0.05$). Only 21 isolates purposively selected across farms and sample types were serotyped. *Salmonella nagoya* was the most prevalent (52%, n=11/21). Other serovars were *Salmonella brijbhumi* (5%, n=1/21); *Salmonella enteritidis* (5%, n=1/21); and *Salmonella enterica* subsp. *enterica* serovar 6, 8: z4 (19%, n=4/21). Four isolates (19%) were untypable. All isolates showed multidrug resistance. Most of the isolates were resistant to ampicillin (82.3%) and tetracycline (76.5%). Some isolates were resistant to cefotaxime (23.5%) and ciprofloxacin (29.4%). The occurrence of multidrug-resistant *salmonella* isolates is considered a critical public health threat that requires urgent global action. There is a need for a coordinated national *salmonella* surveillance program in Nigeria.

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1.Introduction

Non-typhoidal *Salmonella* (NTS) are rod-shaped, motile, gram-negative bacteria. They are one of the most common foodborne pathogens in humans, animals, and the environment (1). *Salmonella* species are responsible for one-third of the global death from foodborne infections (2). Currently, there are over 2500 serotypes of *Salmonella* (3) that causes over 95.1 million cases of gastroenteritis in 2017 (4-5).

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In Africa, foodborne diseases were responsible for 137000 reported deaths (2). In immunocompetent individuals, NTS mostly caused mild (self-limiting) asymptomatic infections. However, using the assemblage of genes in the *Salmonella* pathogenicity island, NTS can cause invasive salmonellosis (6). The case fatality rate of invasive NTS infections in hospitalized patients ranged from 4.1-27% in children (7) and 22-47% in adults (8). Furthermore, co-morbidities such as HIV, tuberculosis, malaria often worsen treatment outcomes of invasive NTS (9-10).

In Nigeria, the abuse and misuse of antibiotics in food animals have led to the emergence of multidrug-resistant *Salmonella* strains (11-16). This has made *Salmonella sp*. a major public health problem (9). Currently, there is no national *Salmonella* surveillance program for animals. However, over the last decade, over 100 serovars of NTS from poultry have been reported across the country by independent researchers. Fagbamila *et al*., (17) reported 79 different NTS serovars and few monophasic variants in a national survey involving twelve states. Raufu et al (12,18,19) reported 11 different serovars of NTS in the North Eastern region of Nigeria.

Human salmonellosis mostly occurs via consumption of contaminated food and water, contact with live animals, and human-to-human transmission, it is essential to improve the access to portable water, improve personal hygiene, waste disposal and ensure optimum environmental sanitation (4). For Nigeria to achieve a drastic reduction in the occurrence of NTS, there is an urgent need to conduct coordinated molecular and spatio-temporal epidemiological studies of NTS using a one-health approach. This will enable proper risk assessment and ensure the creation of critical control points (CCPs) that are necessary for ensuring food safety and the protection of public health (6). Studies conducted by the Center for Science and Environment reported that the commonly detected NTS serovars in poultry were the prevalent serovar in invasive human salmonellosis. This finding emphasizes the threat to human health posed by the colonization of birds by NTS (20).

Poultry products are considered as a major reservoir and source of NTS for humans (1,3). Globally, there are increasing reports of multidrug-resistant Salmonella serotypes in poultry with zoonotic potentials (21-23). Hence, this study aimed to determine the occurrence, circulating serovars and antibiotic resistance profile of NTS isolates from apparently healthy laying birds in Ilorin, Kwara state.

2.Materials and Methods

2.1.Study area, study design, and sample size

The study was conducted in Ilorin, the capital of Kwara State, North Central Nigeria. The city lies within coordinates 8°4799″N and 4.5418°E and has an estimated population of ≈ 1 million people (24). A systematic random sampling of 14 farms from the sampling frame (n=49 farms) within the study area was conducted. The selection of farms was based on the consent of the farmer and their willingness to permit the sampling of their birds. A total of 420 samples were collected. The samples comprised of faeces (n = 140); cloacal swabs (n = 140), feed (n = 70), and water samples (n = 70) which were aseptically collected between January to March 2015. Within a farm, samples from birds, feed and water were collected at random from

different pens, feeding and watering troughs. Only fresh fecal droppings were collected and only healthy birds were sampled.

2.2.Sample collection, isolation, and identification of *Salmonella*

Samples were collected using sterile swab sticks and placed in tubes containing 9 mL of buffered peptone water (Oxoid, Basingstoke, England) and were immediately incubated overnight at 37°C. *Salmonella* was isolated as described by the ISO 6579-1:2017 standards. A loopful of the pre-enriched over-night cultures (from the BPW) were inoculated into fresh Rappaport –Vassiliadis broth (Oxoid, Basingstoke, England) and incubated overnight at 42°C. A loopful (10 µL) of the culture was streaked on Xylose deoxycholate (XLD) agar (Oxoid, Hampshire, UK) and the plate was incubated at 37°C for 24-48 h. The plates were examined for the growth of typical *Salmonella* colonies (red colonies with black centers). All presumptive colonies were purified on nutrient agar (Oxoid Ltd, Hampshire, UK) and biochemical confirmation was conducted using the following tests: triple sugar iron agar, urease agar, citrate, and catalase.

2.3.Serotyping

A purposive selection of 21 confirmed *Salmonella* isolates (from 6 farms) was conducted. These selected isolates were serotyped at the National Veterinary and Agrochemical Research Institute (CODA – CERVA) in Belgium according to the Kauffman-White scheme as described by Raufu *et al* (19).

2.4.Antibiotic susceptibility testing

The Kirby- Bauer disk diffusion method was used for the antibiotic susceptibility testing of isolates. The test was performed by emulsifying 3-5 colonies into a test tube containing 4 mL of 0.9% normal saline to attain turbidity equal to the 0.5% McFarland standard. A sterile cotton swab was used to spread the culture onto a Mueller Hinton Agar (Oxoid, Hampshire, UK). Antibiotic discs (Oxoid, Basingstoke, UK) were placed on agar plates and were incubated overnight at 37°C. *Escherichia coli* ATCC25922 was used as a negative control. The zone of inhibition and the interpretation were based on the latest epidemiological cut-off (ECOFF) set by the European Committee on Antimicrobial Susceptibility Testing (EUCAST - https://mic.eucast.org). The antibiotic disc used (Oxoid Laboratories, UK) and their ECOFF were: gentamicin (30 µg, 16 mm); ampicillin (10 µg, 18 mm); cefotaxime (5 µg, 20 mm); chloramphenicol (30 µg, 19 mm); tetracycline (30 µg, 17 mm); ciprofloxacin (5 µg, not determined). EUCAST does not have an ECOFF for ciprofloxacin to test NTS using the disc diffusion assay. A zone of inhibition greater than the ECOFF is regarded as sensitive while a zone of inhibition lower than the ECOFF is considered resistance.

3.Results

3.1.Prevalence of *Salmonella*

From the 420 samples, a total of 31 (7.4%) *Salmonella* isolates were isolated (Table 1). All isolates were positively confirmed as *Salmonella* sp. on biochemical characterization. Poultry feed had the highest number of *Salmonella* contamination (3.1%, n = 13/420). The

feed also accounted for 42% (n = 13/31) of the total positive *Salmonella* isolates. *Salmonella* isolation rate from fecal, cloacal swabs, and water samples accrued to 32 % (n=10/31), 19.5% (n=6/31), and 6.5% (n=2/31) respectively. There was a significant difference between the total number of positive samples from each farm ($p < 0.05$) (table 2).

Table 1. Frequency of *Salmonella* isolation from samples

Sample type	No. of samples	*Salmonella* positive (%)
Fecal	140	10 (2.4%)
Cloacal	140	6 (1.4%)
Feed	70	13 (3.1%)
Water	70	2 (0.5%)
Total	420	31 7.4%)

3.2.Serotypes and distribution of *Salmonella* serovars in selected farms

The 21 *Salmonella* isolates were serotyped into four (4) different serotypes (Figure 1). *Salmonella nagoya* was the most frequently isolated strain, representing 52% (n = 11) of all serotyped strains. Other serotypes were *S. brijbhumi* (5%, n = 1); *S. enteritidis* (5%, n = 1) and *Salmonella enterica ser.* 6,8: z4 was 19% (n=4). Four Salmonella strains (19%) were untypable. The 21 serotyped Salmonella strains were obtained from 6 different farms (table 2). There was a significant difference between farms ($p<0.05$). Farm 1 was the most contaminated with eight NTS serovars whereas farm 2, 3, and 4 had just one NTS serovar from the fecal droppings and cloacal swabs.

All of the Salmonella serovars were multidrug-resistant (resistant to 3 or more classes of antibiotics) except S. brijbhumi which showed resistance to ampicillin and tetracycline. Most of the NTS serovars were resistant to ampicillin (82.3%, n = 14) and tetracycline (76.5%, n = 13). Cefotaxime had the least resistance with only 4 serovars (23.5%) showing a zone of inhibitions higher than the ECOFF (Table 3). S. enteritidis was resistant to all antibiotics tested except gentamicin and chloramphenicol (Table 3). S. nagoya showed the highest antibiotic resistance profile with a range from 23.5% (ciprofloxacin) to 91% (ampicillin).

Table 2. Distribution of *Salmonella* isolates in sampled farms

	Salmonella serovars			
Farm (Flock size)	*S. nagoya* (6,8; b; 1,5)	*S. brijbhumi* (11; i; 1,5)	*S. enteritidis* (1, 9,12; g,m)	*S. enterica ser. 6,8, z4*
1 (2500)	Feed (n = 5) Feces (n = 2)	-	-	Feed (n = 1)
2 (2000)	Feces (n = 1)	-	-	-
3 (3000)	-	-	-	Feces (n =1)
4 (3000)	-	Cloaca (n = 1)	-	-
5 (3000)	Feces (n = 3)	-	Water (n = 1)	-
6 (1920)	-	-	-	Feed (n =2)

Antibiotic susceptibility testing

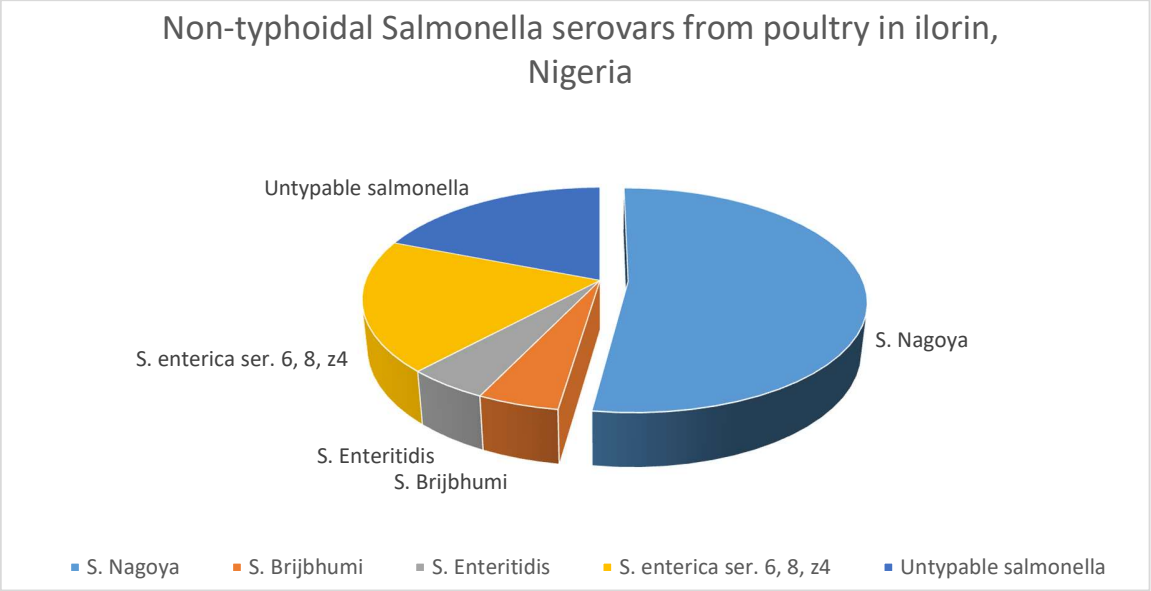

Figure 1. *Salmonella* serovars isolated from poultry in Ilorin, Nigeria.

Table 3. Antimicrobial profiles of *Salmonella* serovars isolated from poultry in Kwara state.

Antibiotic (ECOFF)	*S. nagoya* (n=11, %)	*S. brijbhumi* (n=1, %)	*S. enteritidis* (n=1, %)	*S. enterica ser. 6,8, z4* (n=4, %)
Gentamicin	6 (55%)	0 (0%)	0 (0%)	1 (25%)
Ampicillin	10 (91%)	1 (100%)	1 (100%)	2 (50%)
Cefotaxime	3 (27%)	0 (0%)	1 (100%)	0 (0%)
Chloramphenicol	4 (36%)	0 (0%)	0 (0%)	1 (25%)
Tetracycline	9 (82%)	1 (100%)	1 (100%)	2 (50%)
Ciprofloxacin*	4 (23.5%)	0 (0%)	1(100%)	0 (0%)

*The ECOFF for ciprofloxacin for *Salmonella* sp. has not been determined. Hence, CLSI 2018 breakpoint was used.

4.Discussion

Non-typhoidal *Salmonella* (NTS) sp. are a leading cause of global bacterial gastroenteritis. *Salmonella* causes severe economic loss and poses a serious zoonotic threat to man (11).This study was necessary to identify the circulating *Salmonella* strains in Kwara state. Active surveillance is an integral part of the national action plan for controlling antimicrobial resistance (NAP-AMR). Laying birds, their feed and water all contained Salmonella sp. The prevalence rate of 7.4% was similar to earlier studies reported by Akeem et al (11), Raufu et al (18). However, it is lower than the 10% and 15% reported by Fashae et al (14) and Raufu et al (19) respectively.

The prevalence of *Salmonella* in feed and fecal samples were within the range reported in a country-wide study reported by Fagbamila *et al* (17). The presence of *Salmonella* in feed might be due to the different multiple sources and *S. nagoya* was the most predominant serovar isolated from poultry in this study. It was first reported by humans with acute gastroenteritis in Japan (26). It belongs to the C_2 group (27). This is the first report of *S. nagoya* in Africa. The serovar was reported in animals for the first time in 2011 in an outbreak on a Japanese farm (28). *S. enteritidis* is one of the major causes of human enterocolitis and invasive salmonellosis over the last 20 years (29). The strain is particularly important for its unique ability to contaminate eggs without illness in the affected birds. *S. enteritidis* was responsible for 57.1% of all salmonellosis cases in the EU in 2016 (30). components of poultry feed (11,25). Recently, distinct *S. enteritidis* lineages have been identified with life-threatening diseases in humans (31). *S. enteritidis* in eggs and poultry meat was responsible for 93.9% of all *Salmonella* isolated in the EU between 2014 and 2016 (30). Although *S. enteritidis* was isolated in this study, the prevalence was low (5%, n=1). *S. brijbhumi* is an environmental serovar that was first identified from sewage in India (32). The serovar belongs to the F group (27). Toads are considered to be the natural reservoirs of this strain of *Salmonella* (33). Monophasic *S. enterica subsp. enterica ser. 6,8; z4* was also isolated from this study. While many monophasic variants have been greatly studied, others such as those isolated from this study are novel and further studies are needed to molecularly characterize them. Akeem et al., (11) also reported 4 different monophasic *Salmonella* variants. The occurrence of *S. nagoya, S. brijbhumi, S. hududdify, S. agama*, and other relatively new serovars in Nigeria, could be indications for poor sanitation, use of poultry droppings as organic fertilizer, international travels, porous borders, gaps in importation controls, poor handling and contamination of poultry feed along the production chain by rodents and other pests.

Most of the Salmonella serovars were multidrug resistant. As anticipated, resistance to tetracycline (76.5%) and ampicillin (82.3%) was very high. This is in agreement with previous studies reported by Akeem et al., (11); Raufu et al., (18); Fashae et al., (14); Adeyanju, (13); Thomas et. al., (34). Most serovars (64.7%) were sensitive to gentamicin. Although resistance to cefotaxime and ciprofloxacin were low, they are considered important because third-generation cephalosporins and quinolones are critical human drugs that are strategic in the treatment of invasive Salmonellosis and MDR pathogens (35,36). The production of extended-spectrum beta-lactamases conferring cephalosporin resistance especially in Enterobacteriaceae is categorized as an urgent global

public health threat (37). The low resistance to cefotaxime was similar to report by Raufu et al., (18) but contrary to the findings of Akeem et al., (11); Agada et al., (38); Vincent et al., (39) where most non-typhoidal Salmonella serovars were resistant to cefotaxime. The resistance to ciprofloxacin (29.4%) observed in these serovars was lower than previously reported (9,11,13,15,18,40).

All over the world, poultry and its product have been a major source of human salmonellosis. Factors such as indiscriminate antibiotic use by most farmers, poor utilization of veterinary services, poor management practices, and inefficient biosecurity measures by most small and middle scale poultry farmers as well as the use of poultry droppings as organic fertilizer for crop cultivation have been associated with increased prevalence of multidrug-resistant Salmonella strains in Nigeria (17,41).

To further evaluate the zoonotic potential of these rare serovars, whole-genome sequencing analysis of these isolates would give an in-depth view of the vast genomic diversity of NTS. Using the appropriate bioinformatic pipeline, these isolates should be compared with NTS in humans to assess the risk of NTS in poultry to human health.

5.Conclusion

This study reported the first occurrence of two rare, MDR *Salmonella* serovars in healthy laying birds in Ilorin, Nigeria. These pose threats to food safety and public health. There is an urgent need to improve active *Salmonella* surveillance programs and implement the national action plan on AMR to regulate antibiotic usage in Nigeria. Furthermore, there is a need to create awareness among poultry farmers.

Conflict of interest

The authors declare that they have no conflict of interest.

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