



Isolation and genomic characterization of “unassigned” *Salmonella enterica* serovars from poultry in Ilorin, north-central Nigeria

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Abstract

Salmonellosis is an important global foodborne disease caused by *Salmonella enterica* (*S. enterica*). Strains that are resistant to a variety of antibiotics were known to constitute major hazard to public health. The objectives of this study are to determine the serovar distributions, genomic antimicrobial resistance, prediction of genes conferring resistance to selected antibiotics, the multi-locus sequence typing (MLST), and plasmid replicon typing of “unassigned” *S. enterica* isolated from poultry. A total of 300 samples comprised of: post-mortem tissues (n = 150), cloacal swabs (n = 30), and poultry environment (n = 120) were aseptically collected and analyzed between January and June, 2017. Presumptive *S. enterica* isolates were characterized using conventional cultural methods, biochemical tests, and serotyping. The isolates were characterized, using Whole Genome Sequencing (WGS) Method. Five “unassigned” *S. enterica* serovars were recovered from four matrices (liver, n = 1; water, n = 1; cloacal swab, n = 2; poultry feed, n = 1). Prediction of point mutation in *parC* (T57S) was reported in two strains which confer resistance to nalidixic acid; in addition to this, prediction of *fosA7* that confers resistance to fosfomycin was identified in one of these strains. Three isolates each encoded plasmid mediated quinolone resistance (PMQR) *qnrB69* and *bla-CMY-98* genes expected to confer decreased susceptibility to ciprofloxacin and resistance to ampicillin, amoxicillin-clavulanic acid, cefoxitin, and ceftriaxone, respectively. Three sequence types, ST-6111, 6114 and 7073 were detected. None of the isolates harbored plasmid replicon. This study highlights the importance of “unassigned” *S. enterica* serovars in the emergence and spread of *S. enterica* in poultry. There is a need for the establishment of national collaborative *Salmonella* program to further investigate the pathogenic and public health risk to humans, of “unassigned” *S. enterica* serovars in Nigeria.

Keywords: *bla*-*CMY*-98 gene; *fosA* gene; MLST; Poultry; *qnrB69* gene; *S. enterica*

Introduction

Salmonella has been incriminated as an important contributor to global foodborne illness with poultry and other foods of animal origin being considered as reservoirs of *Salmonella*. Infections of humans with non-typhoidal *Salmonella* are commonly linked to the consumption of undercooked foods, including poultry products (Foley *et al.*, 2011). *S. enterica* serovars that are commonly incriminated in poultry infect a broader spectrum of hosts and also cause infection in humans (Gast, 2007).

Interestingly, some isolates are assigned antigenic formulae without serovars name, such isolates, described as “unassigned serovar” in the context of this paper, are often not always well investigated for their roles in food animal salmonellosis and public health safety.

The reported annual incidence of human salmonellosis globally is approximately 93.8 million cases (Majowicz *et al.*, 2010) and poultry products are particularly being incriminated as vehicles of *Salmonella* transmission leading to substantial economic losses, including human and animal diseases.

Globally, foodborne infections are under-reported but the burden is further exacerbated in developing countries like Nigeria where *S. enterica* infections and diseases are often grossly under-reported or totally not reported, this makes it difficult to evaluate the magnitude of the problem (Barbour *et al.*, 2015). The under- or lack of reporting of salmonellosis in developing countries is of concern especially where diagnostic facilities and qualified personnel are inadequate or totally absent, this makes disease surveillance and source attribution a challenge (Barbour *et al.*, 2015).

Reports have shown that majority of the commonly isolated *S. enterica* serovars in poultry in a given geographic location are among the frequently reported serovars associated with human salmonellosis, thus, corroborating the roles of *Salmonella* in poultry farms colonization and a possible threat to public health (Foley *et al.*, 2008). Unfortunately, the pathogenic roles of “unassigned” *S. enterica* serovars are usually undermined and the antimicrobial, genomic characteristics, pathogenicity potentials or virulence capabilities of this category of *S. enterica* are usually not investigated as compared to the commonly encountered or known *S. enterica* serovars during outbreak or surveillance (Jibril *et al.*,

2021). Therefore, to ascertain the actual *Salmonella* serovars distribution in humans and foods of animal origin, *Salmonella* epidemiological trends in the country need to be understood.

Since non-typhoidal *Salmonella* illness is self-limiting, antimicrobial therapy is usually not prescribed except in severe cases of invasive salmonellosis complications. Beta-lactam and quinolone antimicrobials are the drugs commonly prescribed as a first line drug of choice; however, over the years, resistance to these antimicrobials has increased among the non-typhoidal *Salmonella* isolates from both humans and food animals (Wong *et al.*, 2014). Fluoroquinolones antibiotics are important for treatment of infections in both humans and animals; resistance to these antibiotics can lead to treatment failure (Gagliotti *et al.*, 2008; Threlfall *et al.*, 2008). In *Enterobacteriaceae*, (fluoro) quinolone resistance is mainly due to point mutations in the quinolone resistance-determining region (QRDR) of gyrase (*gyrA* and *gyrB*) and topoisomerase (*parC* and *parE*) genes. The frequently reported high level resistance to ciprofloxacin is associated with double alterations in the *gyrA* gene and single or double mutations in *parC* gene (Redgrave *et al.*, 2014). Over the years, there have been numerous reports on the occurrence of plasmid mediated quinolone resistance (PMQR) harbored by several *qnr* genes which prevented bacterial topoisomerases from the actions of quinolones, because they harbored pentapeptide protein, even though they eventually do not induce high level resistance, their presence only enhances mutation in the QRDR (Robicsek *et al.*, 2006). At present, there is paucity of documented literature on farm level prevalence and distribution of these “unassigned” non-typhoidal *S. enterica* serovars in poultry farms in Nigeria; therefore, molecular characterization of these strains for evaluation of their pathogenicity and public health significance will assist in providing a baseline information for future study. This study aimed to determine the sequence types using MLST, antimicrobial resistance genes, virulence genes and plasmids of “unassigned” *S. enterica* isolates obtained from poultry farms in Ilorin, north-central Nigeria.

Materials and Methods

Sample collection and preparation

Three hundred samples comprised of cloacal swabs, poultry feed, litter, water from troughs, post-mortem tissues from dead chickens; liver, spleen, caeca, heart, and intestine (one sample per bird, if any) were randomly collected from ten commercial poultry farms in Ilorin metropolis, Nigeria from January to June, 2017. Each farm was visited three times for sampling, all the farms were visited on each sampling day. Thirty samples were collected aseptically per farm using sterile sample bottles and transported to the laboratory in boxes containing ice packs for analysis (Table 1).

Isolation and identification of Salmonella species

Samples were dispensed into buffered peptone water (Oxoid, Hampshire, UK) at a ratio of 1 in 10 (sample to broth) and incubated aerobically for 20 - 24 hours at 37°C ± 2. One milliliter of the pre-enriched broth culture was inoculated into 9.0 ml of Selenite-F broth (Fluka Biochemika, Steinheim, Germany) and incubated aerobically for 20 - 24 hours at 37°C ± 2. The broth was streaked on *Salmonella Shigella* agar (Laboratorios Britania, Buenos Aires, Argentina) and incubated for 20 - 24 hours at 37°C ± 2. Dark discrete colonies on *Salmonella Shigella* Agar were streaked on selective media, Xylose Lysine Deoxycholate (XLD) agar (Laboratorios Britania, Buenos Aires, Argentina) and incubated aerobically at 37°C ± 2 for 20 - 24 hours. Presumptive *Salmonella* isolates with pink/red colonies and dark center were confirmed by using the following biochemical tests: Voges-Proskauer, methyl red, citrate utilization, triple sugar iron agar, urease, oxidase, and hydrogen sulfide and interpreted according to Feltham and Barrow (2003), streaked on nutrient agar slant and incubated at 37°C ± 2 for 20 - 24 hours. The presumptive isolates were

subsequently stored in the refrigerator for serotyping and further analysis.

All the isolates were serotyped at the WHO National Salmonella and Shigella Reference Center, Bangkok, Thailand, by slide agglutination, O and H antigens were characterized by agglutination with hyperimmune sera (S & A Reagents Laboratory, Limited, Bangkok, Thailand), and serotypes were assigned according to the White-Kauffmann-Le Minor scheme as previously described by Raufu *et al.* (2009).

Whole Genome Sequencing data collection

Out of the 300 samples serotyped, five “unassigned” *S. enterica* isolates were shipped on nutrient agar slants to U.S. Food and Drug Administration, College Park, Maryland, USA for whole genome sequencing. Bacterial DNA extraction was performed on the overnight cultures using Dneasy blood and tissue extraction kit (Qiagen, Valencia, CA, USA) in accordance to the manufacturer’s instructions. Sequencing libraries were constructed by using 0.2 ng/μl of prepared DNA using the Nextera XT DNA library prep kit (Illumina, San Diego, CA, USA). Sequencing was executed on the MiSeq Illumina instrument with the 500-cycle MiSeq reagent V2 kit (2 × 250 bp) according to the manufacturer’s instructions. Raw data were promptly uploaded to FDA’s GenomeTrakr database (Allard *et al.*, 2016), hosted within NCBI Pathogen Detection (NCBI Resource Coordinators, 2017) under the bioproject PRJNA186035.

Whole Genome Sequence analysis

Raw reads were subjected to genome assembly using SPAdes version 3.8 (Bankevich *et al.*, 2012), while genome annotation was performed with the NCBI Prokaryotic Genome Annotation Pipeline

Table 1. Types and number of samples obtained from poultry farms in Ilorin, North-central Nigeria

Source	Samples	
	Types	No. (per farm)
Post-mortem	Liver	30
	Spleen	30
	Caecum	30
	Heart	30
	Intestine	30
Apparently healthy birds	Cloacal swabs	30
Poultry environment	Feed	30
	Water	30
	Litter	30

(PGAP) (Tatusova *et al.*, 2016). *Salmonella* serotyping by WGS was predicted with the SeqSero version 2.0 software tool (Zhang *et al.*, 2015).

Resistance determinants in the ResFinder and PointFinder databases were identified in assemblies using starAMR v. 0.4.0. Plasmid genes were identified using abricate v. 0.8.10 (Seemann, 2015) and a modified Version of PlasmidFinder database (Zankari *et al.*, 2012). Predicted resistance phenotypes were assigned by using the determinants detected and the ResFinder and PointFinder drug keys developed by the Centers for Disease Control and Prevention (Feldgarden *et al.*, 2019).

Multilocus sequence typing (MLST) was carried out using WGS data using the sequence of seven housekeeping genes: *aroC*, *dnaN*, *hemD*, *hisD*, *purE*, *sucA*, and *thrA*. Genome assemblies were uploaded to the Centre for Genomic Epidemiology MLST 2.0 tool (Larsen *et al.*, 2012) to assign sequence types (STs) to each study isolate based on the set of alleles derived from the aforesaid seven loci.

Results

Out of the 300 samples, five were positive for *S. enterica* after conventional serotyping and SeqSero 2 evaluation, these include *S. enterica* serovar 45: d: 1, 7 (n = 3); *S. enterica* serovar 4: z: -; and *S. enterica* serovar -: d:1,7. The five isolates were from farms A2, A6, and A9, they were obtained from water, drinking trough, liver, cloacal swabs from apparently healthy chicken, and poultry feed (Table 2).

Three sequence types, ST-6111, ST-6114, and ST-7073 were obtained. The predominant sequence types are ST-6111 (n = 3) from liver, water and cloacal swabs. It was observed that cloacal swab isolate with ST-6111 did not share similar antigenic formula with the isolates obtained from liver and water despite having similar sequence type. Sequence types 6114 and 7073 were obtained from cloacal swab and poultry feed respectively (Table 3).

The two *S. enterica* serovar 45:d:1,7 isolates from liver, water, and *S. enterica* serovar -:d:1,7 isolates from cloacal swab both harbored plasmid mediated

quinolone resistance (PMQR) gene *qnrB69* with expected low level susceptibility to ciprofloxacin. Additionally, they harbored a CMY beta-lactamase gene, *bla-CMY-98* which confers resistance to ampicillin, amoxicillin-clavulanic acid, ceftiofur, and ceftriaxone (cephalosporin) antimicrobials. *S. enterica* serovar 4:z:- and *S. enterica* serovar 45:d:1,7 isolates from cloacal swab and poultry feed respectively both harbored a previously described *parC*(T57S) mutation, this mutation usually cannot independently confer quinolone resistance (Baucheron *et al.*, 2005). In addition, *S. enterica* serovar 45:d:1,7 isolates obtained from feed harbored a chromosomal gene, *fosA7* which conferred a high level of resistance to fosfomycin antimicrobial. Plasmid was not detected from all the isolates (Table 3).

Discussion

In recent times, “unassigned” *S. enterica* serovars are increasingly being isolated (Switt *et al.*, 2009). In Nigeria, Raufu *et al.* (2013) and Ahmed *et al.* (2019) reported “unassigned” serovars from poultry in Maiduguri and Ilorin respectively. Similarly, Fashae & Hendriksen (2013) as well as Fashae & Hendriksen (2018) also reported “unassigned” *S. enterica* serovars from humans, food animals and poultry respectively. Furthermore, same categories of *Salmonella* were reported in Thailand by Pornruangwong *et al.* (2008), Huoy *et al.* (2014) and in Japan by Kurosawa *et al.* (2012) and Ido *et al.* (2014). The occurrence of these “unassigned” *S. enterica* serovars in foods of animal origin constitutes a threat to animal husbandry and a potential risk to public health, because the pathogenicity of these “unassigned” strains is similar to the commonly reported *S. enterica* serovars (Crayford *et al.*, 2014).

In this study, *S. enterica* serovars were isolated from all the three major sources investigated; post-mortem, apparently healthy birds, poultry environment, and also across the sample matrices comprised of liver, water, cloacal swab and poultry feed, this corroborated similar study in Ethiopia.

Table 2. Occurrence of “unassigned” *Salmonella* serovars isolated from poultry farms in Ilorin, north-central Nigeria

Serial No	Farm ID. No.	Sample types	Date of collection	Serovars
1.	A2	Water	2017	45:d:1,7
2.	A6	Liver	2017	45:d:1,7
3.	A6	Cloacal swab	2017	4:z:-

4.	A9	Cloacal swab	2017	-:d:1,7
5.	A9	Farm feed	2017	45:d:1,7

Table 3. Serovars, resistance genes, plasmid and MLST profiles of “unassigned” *S. enterica* isolated from poultry farms in Ilorin, North-central Nigeria

Source	NCBI-Biosample	SRA Run	SeqSero 2(JC)	MLST	Resistance Determinants			Plasmids	Predicted Resistance
					Mutation	PMQR	Gene		
Liver	SAMN11896308	SRR9163920	45:d:1,7	6111	None	<i>qnrB69</i>	<i>bla-CMY-98</i>	None	Ampicillin, Amoxicillin-Clavulanic Acid, Cefoxitin, Ceftriaxone, Ciprofloxacin (I/R)
Water	SAMN12601202	SRR9994407	45:d:1,7	6111	None	<i>qnrB69</i>	<i>bla-CMY-98</i>	None	Ampicillin, Amoxicillin-Clavulanic Acid, Cefoxitin, Ceftriaxone, Ciprofloxacin (I/R)
Swab	SAMN11896213	SRR9163753	-:d:1,7	6111	None	<i>qnrB69</i>	<i>bla-CMY-98</i>	None	Ampicillin, Amoxicillin-Clavulanic Acid, Cefoxitin, Ceftriaxone, Ciprofloxacin (I/R)
Swab	SAMN11897672	SRR9164980	4:z:-	6114	<i>ParC(T57S)</i>	None	None	None	None
Feed	SAMN12601670	SRR9998283	45:d:1,7	7073	<i>ParC(T57S)</i>	None	<i>fosA7</i>	None	Fosfomycin

which reported *Salmonella* contamination of poultry and poultry environment (Abdi *et al.*, 2017), thereby classifying *S. enterica* as a major zoonotic pathogen of public health significance. The occurrence of *S. enterica* in this study tends to corroborate the belief that human and animal health are threatened by *S. enterica* implication in food of animal origin (Ahmed *et al.*, 2019).

The isolation rate from this study (1.7 %) is higher when compared to 0.2% reported by Raufu *et al.* (2013) and 0.8% reported by Fagbamilu *et al.* (2017), thus suggesting chickens and poultry environments as important reservoirs for *S. enterica* in Nigeria. The overall frequency of *Salmonella* from cloaca swabs in this study was 0.7%, this is lower than 8% reported by Ahmed *et al.* (2019) and Ammar *et al.* (2016) in Nigeria and Algeria respectively; however, it is in agreement

with 0.9% reported in Trinidad and Tobago by Adesiyun *et al.* (2014).

The reason for the observed variations may be linked to differences in biosecurity practices and management system on the farm leading to serotype variations between farms, furthermore the variations may also be due to environmental persistence of *Salmonella* which facilitates its epidemiology in poultry (McIlroy *et al.*, 1989).

Salmonella resistance in poultry remains a controversial issue due to its impact on food production, animal welfare and public health. The irrational administration or overuse of antimicrobials on farms are the major factors responsible for the introduction of antimicrobial resistance genes to the food chain (Davis *et al.*, 2011).

Resistance to antimicrobials, including the third-generation cephalosporin and fluoroquinolone

antibiotics were reported in this study. It is generally believed that one of the reasons for the increased prevalence of antimicrobial-resistant strains in food-producing animals is the usage of antimicrobials in animal husbandry and poultry practices.

The findings in our study is worrisome, this is due to the observed level of resistance genes among the majority of the isolates. This observation could be due to indiscriminate use of non-prescription antimicrobials in the veterinary and public health sectors (Beyene *et al.*, 2015; Okeke *et al.*, 2005). Furthermore, there is lack of compliance and monitoring of antimicrobials at all levels in Nigeria farming sector.

In addition, administration of antimicrobials at sub-therapeutic or prophylactic doses in food animals could encourage on-farm selection for antimicrobial resistance genes in *Salmonella* and other potential human and animal pathogens. The incorporation of antibiotics in animal diets at sub-therapeutic levels to serve as prophylaxis and/or growth promoters has contributed to the development of resistant strains (Shah & Korejo, 2012).

Quinolone resistance in *Salmonella* is known to be mediated by mutation of QRDRs of *gyrA*, *gyrB*, *parC*, and *parE* and/or the acquisition of PMQR genes (Egualé *et al.*, 2017). We reported two isolates from feed and cloacal swab that showed single mutation at *parC*(T57S); this mutation usually does not independently confer quinolone resistance, *parC* mutations are usually associated with reduced susceptibility to fluoroquinolone antibiotics such as ciprofloxacin; however, the *parC* T57S mutation is not known to confer high-level resistance alone which corroborated previous studies (Redgrave *et al.*, 2014). The three isolates obtained from liver, water, and cloacal swab harbored a beta-lactamase (*bla*-*CMY*-98) resistance gene with anticipated resistance to ampicillin, amoxicillin-clavulanic acid ceftiofur, and ceftriaxone. In addition, they encoded PMQR gene, *qnrB69* commonly associated with decreased susceptibility to fluoroquinolones.

Several studies have implicated *fosA* gene to be responsible for fosfomycin resistance, and are usually located on the plasmid to enhance its rapid dissemination as a mobile genetic element (Poirel *et al.*, 2018; Yao *et al.*, 2016), but recently, Hou *et al.* (2012) reported that the major type of genes conferring resistance to fosfomycin appears to be chromosomal rather than plasmid-mediated, this is in tandem with the outcome of our study. In addition, the isolate from feed harboured *parC*(T57) mutation is not known to cause resistance alone, while the *fosA7* gene

is anticipated to enhance the strain ability to develop resistance to fosfomycin.

We reported three different sequence types in this study: ST-6111, 6114, and 7073. To the best of our knowledge, this is the first report of these sequence types in these “unassigned” *S. enterica* serovars because they are uncommon serovars. Among the three isolates with ST-6111, the isolates from cloacal swab with different antigenic formula had similar sequence types and antimicrobial profiles with the isolates from liver and water, suggesting that they are genetically related, but further investigation would be needed to confirm this speculation.

This study reports for the first-time genomic characterization of some “unassigned” *S. enterica* serovars obtained from poultry. The detection of three resistance genes coupled with mutation of the quinolone resistance-determining regions (QRDRs) are of public health significance because these antimicrobials are used for clinical chemotherapy in human and veterinary practices. More worrisome is the *bla*-*CMY*-98 gene in some of the isolates because plasmids carrying *fosA*-genes commonly harbor additional resistance genes that foster the chance of co-selection fosfomycin resistance under the selective pressure by other antimicrobials (Poirel *et al.*, 2018). Our finding provides useful baseline information that will benefit future researchers, public health workers, and farmers. The study showed that the hitherto unrecognized *S. enterica* serovars are a risk to public health and poultry production; they also contributed to the spread and emergence of “unassigned” *S. enterica* in Ilorin, north-central Nigeria.

The limitation in this study is the non-detection of virulence genes. Evaluation of virulence genes would complement this study's outcome and clarify the serovar's pathogenicity potential and subsequent evaluation of its public health importance. It is recommended that further work should be carried out to evaluate the potential virulence profiles encoded in the various virulence loci.

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Conflict of interest

The authors declare that there is no conflict of interest.

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