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Phenotypic and molecular detection of multi-drug resistant *Salmonella* Enteritidis, *Salmonella* Typhimurium and *Salmonella* species in retail raw beef and chicken

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ABSTRACT

Objective: To detect *Salmonella* species and its' serovars, *Salmonella* Enteritidis (*S.* Enteritidis) and *Salmonella* Typhimurium (*S.* Typhimurium) in retail raw beef and chicken in Nigerian markets.

Methods: A total of 100 samples, including 50 beef and 50 chicken were purchased from retailers in Lagos, Nigeria. Presence of *Salmonella* species and its' serovars, *Salmonella* Enteritidis and *Salmonella* Typhimurium in the beef and chicken were assessed phenotypically and by PCR assay. Antibiotic susceptibility test of the isolates was done. Primers were from *fli*C and *sef*A genes of *Salmonella* Typhimurium and *Salmonella* Enteritidis, respectively.

Results: From PCR analysis, *fli*C gene (559 bp) was amplified in one beef sample and was positive for *S.* Typhimurium while *sef*A gene (312 bp) was amplified in one chicken and three beef samples and were positive for *S.* Enteritidis. In all, 1% of total meat had *S.* Typhimurium while 4% and 18% were contaminated with *S.* Enteritidis and *Salmonella* spp., respectively. For beef, 2% was contaminated with *S.* Typhimurium, 6% with *S.* Enteritidis and 26% with *Salmonella* spp. In chicken 2% was contaminated with *S.* Enteritidis, 12% with *Salmonella* spp. A total of 23 isolates were obtained by ERIC-PCR. All *Salmonella* spp. were 100% resistant to amoxicillin and amoxicillin-clavulanate. Strains of *S.* Enteritidis were also resistant to cotrimoxazole, nitrofurantoin, nalidixic acid, gentamycin and tetracycline.

Conclusions: The presence of *S.* Typhimurium, *S.* Enteritidis and *Salmonella* spp. in retail raw beef and chicken and their multi-drug resistance is of health significance and great concern because the two serovars are commonly implicated in human salmonellosis.

1. Introduction

Globally, *Salmonella* is a major cause of foodborne diseases[1,2]. The incidence of non-typhoidal *Salmonella* is estimated at 1.3 billion cases with annual death rate of 3 million[3]. It results in morbidity, mortality and great economic loss[4,5]. Human salmonellosis is most frequently caused by *Salmonella* Typhimurium (*S.* Typhimurium) and *Salmonella* Enteritidis (*S.* Enteritidis)[6]. Among the over 2 500 serovars identified within *Salmonella enterica* subspecies *enterica*, *S.* Typhimurium continues to be one of the most frequently recovered from food animals worldwide[7]. Due to its broad host range, *S.* Typhimurium is also one of the most common serotype isolated from human clinical cases of food-borne salmonellosis. Poor sanitary conditions have been identified to be responsible for the transmission of *Salmonella* spp., *S.* Typhimurium (group D) and *S.* Enteritidis (group B) in developing countries. In sub-Saharan Africa, they have been reported to be the cause of 79%–95% of all bacteriaemic non-typhoidal *Salmonella* infections or foodborne outbreaks[8,9], and are associated with case fatality rate of 20%–25%[10].

Salmonella can be transmitted to humans from animals and by consuming foods from animal sources such as milk, egg, poultry meat and beef which serve as reservoirs[11,12]. During the production of meat, the major source of *Salmonella* contamination of carcasses is the evisceration stage in slaughter house[13]. In order to ensure food safety and for the purpose of food borne disease surveillance, foods should be examined routinely for the presence of *Salmonella*. Conventional typing methods such as, biotyping, serotyping and phage typing which are based on phenotypic characteristics have been used extensively for this purpose[14]. However, they are less discriminative. Molecular typing methods offer higher discrimination[14] and have been employed for identification of *Salmonella* spp.[9].

Studies on the molecular typing of microbial isolates have

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centred on the use of PCR and macro restriction mediated analysis of microbial DNA. For microbial population structures to be well defined, typing techniques are very important and those being used in recent time are those generally based on genomics and in particular comparative genomics[15]. Monzur *et al*.[16] in their study selected primers for the presence of *inv*A and *fli*C genes of *Salmonella* spp. and *S.* Typhimurium, respectively that they further used in the confirmation of *Salmonella* serovars from chicken eggs.

Mengistu *et al*.[17] carried out a study in which they isolated, identified and used PCR to detect and confirm *Salmonella* species from poultry materials which were collected from the field, and among the confirmed isolates were *Salmonella* Heidelberg, *Salmonella* Essen, *S.* Typhimurium, *Salmonella* Kastrup and *Salmonella* Ayinde. The PCR amplification of suspected *Salmonella* isolates resulted in a product of approximately 550 bp and proved that this tool can be used efficiently for the rapid detection of *Salmonella* organisms. Tsen[18] demonstrated the processes involved in molecular typing of *Salmonella enterica* serovars: Typhi, Typhimurium and Enteritidis which were isolated in Taiwan. In his study, it was observed that adequate genetic diversity occurred in *Salmonella* Typhi and *S.* Typhimurium strains, while great genetic similarity was established in *S.* Enteritidis. In addition, the most prevalent and widely spread strains of *S.* Enteritidis and *S.* Typhimurium were identified using PCR methods as well as PFGE and plasmid profiles. Smith *et al*.[19] conducted a study in Nigeria on molecular typing of *Salmonella* spp. isolated from food handlers and animals. They concluded that RAPD1 and RAPD2 primers gave the most discriminatory patterns and recommended their usefulness for epidemiological typing of *Salmonella* spp. in Nigeria.

Salmonella is a major cause of food borne illness with *S.* Typhimurium and *S.* Enteritidis being very predominant. *Salmonella*, particularly antibiotic resistant strains if present in food animals can be transmitted to humans. Consumers may therefore face the hazard of this pathogen in their meat especially if not well cooked. The aim of this research therefore is to isolate and identify *S.* Typhimurium, *S.* Enteritidis and *Salmonella* spp. present in raw beef and chicken on retail in Nigerian markets using phenotypic and molecular methods as well as screen for their antibiotic susceptibility.

2. Materials and methods

2.1. Sample collection

One hundred samples of raw meat (50 of beef and 50 of chicken) were purchased from five markets, each located in Ago (samples A), Cele (samples C), Oshodi (samples O), Mushin (samples M) and Yaba (samples Y), Lagos State, Nigeria. Ten samples each of beef and chicken were collected from each of the five markets. Beef samples were not frozen, while the chicken samples were frozen previously but thawed as they were all displayed for sale on tables by the retailers. Samples were collected in labeled polyethylene bags and transported to the laboratory in ice packs.

2.2. Isolation of Salmonella

From each sample, 25 g was weighed and homogenized in 225 mL of buffered peptone water and incubated at 37 ˚C for 24 h. Aliquots (0.1 mL) of each homogenate was inoculated into 9.9 mL of Rappaport-Vassiliadis *Salmonella* broth for enrichment. They were incubated at 41.5 ˚C for 24 h. A loopful of the broth culture was streaked on Xylose-Lysine Deoxycholate agar plates and incubated at 37 ˚C for 24 h. Developed colonies were sub-cultured to obtain pure cultures.

2.3. Characterization and identification of isolates

The isolates were identified using cultural characteristics, colonial morphology, and API 20E test kit by Bio Merieux SA which was used to carry out biochemical tests. The kit was prepared according to the manufacturer's specification. Isolates were serotyped using PCR technique and genotyped by enterobacterial repetitive intergenic consensus (ERIC)-PCR.

2.4. DNA extraction from isolates

The DNA extraction from the isolates was carried out according to the boiling method of Zolan and Pukkila[20].

2.5. PCR

The extracted DNA was subjected to PCR assay. This was to further identify the serological variants (serovars), *S.* Typhimurium and *S.* Enteritidis amongst the isolates. Two pairs of primers were used. The Fli 15 and Tym primers specific for the *fli*C gene of *S.* Typhimurium[21] and sef 167- sef 478 primers specific for the *sef*A gene of *S.* Enteritidis[22].

ERIC-PCR amplification was carried out using single primer ERIC1 (5' TGAGCATAGACCTCA 3'). The 25 μL reaction mixture consisted of 1× PCR buffer, 1.5 mm magnesium chloride 200 μm of each dNTP, 20 pmol of the primer and 1 IU Taq DNA polymerase. Amplification was carried out in an Eppendorf master cycler gradient using the following cycling parameters. An initial denaturation at 95 ˚C for 5 min followed by 40 cycles at 95 ˚C for 30 s, 36 ˚C for 1 min and 72 ˚C for 1 min 30 s. This was followed by a final extension at 72 ˚C for 10 min.

The PCR was separated on a 1% agarose gel and a 100 bp DNA ladder was used as DNA molecular weight marker.

2.6. Antimicrobial susceptibility testing

Susceptibility of isolates to antibiotics was determined on Muller-Hinton agar by the disc diffusion method[23]. The antibiotics discs (Oxiod, UK) used were amoxicillin (25 μg), cotrimoxazole (25 μg) nitrofurantoin (30 μg), gentamycin (10 μg), nalidixic acid (30 μg), ofloxacin (30 μg), amoxicillin-clavulanate (20/10 μg) and tetracycline (30 μg). The plates were incubated at 37 ˚C for 18–24 h. The diameter of zones of inhibition was measured and interpreted according to Clinical and Laboratory Standarts Institude[24] chart.

3. Results

From the 100 meat samples analysed, 23 samples (23%) were contaminated with presumptive *Salmonella* colonies on Xylose-Lysine Deoxycholate agar plates which appeared as red colonies with dark center. After identification and serotyping, it was discovered that out of the 23 samples, 1% was contaminated with *S.* Typhimurium, 4% with *S.* Enteritidis and 18% with *Salmonella* spp. (Table 1). From the 50 beef samples, 2% was contaminated with *S.* Typhimurium, 6% with *S.* Enteritidis and 26% with *Salmonella* spp. (Table 1). For chicken, 2% was contaminated with *S.* Enteritidis,

12% with *Salmonella* spp. (Table 1). The result of the PCR analysis showed that the *fli*C gene (559 bp) was amplified in isolate OB4 (*S*. Typhimurium) while the *sef*A gene (312 bp) was amplified in isolates MB4, OC6, YB3 and YB5 (*S*. Enteritidis). Gel pattern indicated similar and divergent strains polymorphisms.

Table 1
Occurrence of *S*. Enteritidis, *S*. Typhimurium and *Salmonella* spp. in beef and chicken.

Bacteria	Occurrence (%)		
	Beef (n = 50)	Chicken (n = 50)	Total meat (n = 100)
S. Enteritidis	6	2	4
S. Typhimurium	2	–	1
Salmonella spp.	26	12	18

–: None.

All the 23 (100%) *Salmonella* isolates were resistant to amoxicillin and amoxicillin-clavulanate and susceptible to oflaxacin. All strains of *S*. Enteritidis isolated from beef displayed resistance to at least five antibiotics which included amoxicillin, cotrimoxazole, nitrofurantoin, amoxicillin-clavulanate and tetracycline. Two of these isolates were in addition resistant to nalidixic acid and one of the two to gentamycin. The only isolate of *S*. Enteritidis from chicken displayed resistance to two antibiotics, *viz*. amoxicillin and amoxicillin-clavulanate. One isolate of *S*. Typhimurium was recovered from beef and it was resistant to amoxicillin and amoxicillin-clavulanate (Table 2). Of the 23 isolates, 26.09% were resistant to cotrimoxazole and tetracycline, 39.13% to nitrofurantoin, 13.04% to gentamycin and 8.70% to nalidixic acid.

Table 2
Antimicrobial susceptibility of *Salmonella* isolated from retail raw beef and chicken.

S/No	Isolates	Serovars	AMX 25 μg	COT 25 μg	NIT 300 μg	GEN 10 μg	NAL 30 μg	OFL 30 μg	AMC 30 μg	TET 30 μg
1	AB1		R	S	S	I	S	S	R	I
2	AB2		R	S	S	I	S	S	R	I
3	AB3		R	S	R	I	I	S	R	I
4	AB7		R	S	I	I	I	S	R	I
5	AB8		R	S	S	R	I	S	R	S
6	AB9		R	S	R	I	S	S	R	S
7	AC1		R	S	S	I	S	S	R	I
8	AC4		R	S	S	I	I	S	R	I
9	AC6		R	I	S	I	S	S	R	S
10	MB1		R	S	I	S	S	S	R	I
11	MB4	*S*. Enteritidis	R	R	R	S	I	S	R	R
12	MB5		R	I	S	I	S	S	R	I
13	MB6		R	I	I	I	S	S	R	S
14	MB8		R	S	I	I	S	S	R	S
15	MC1		R	R	R	I	S	S	R	S
16	CC3		R	R	R	R	S	S	R	R
17	CC6		R	R	R	I	S	S	R	R
18	OB4	*S*. Typhimurium	R	I	S	I	I	S	R	S
19	CB8		R	I	R	S	S	S	R	R
20	OB6		R	S	S	S	S	S	R	I
21	OC6	*S*. Enteritidis	R	S	S	S	I	S	R	I
22	YB3	*S*. Enteritidis	R	R	R	R	R	S	R	R
23	YB5	*S*. Enteritidis	R	R	R	S	R	S	R	R

R: Resistant; I: Intermediate; S: Susceptible; AMX: Amoxicillin (> 20 = S, < 19 = R and 19–20 = I); COT: Cotrimoxazole (> 19 = S, < 15 = R and 15–19 = I); NIT: Nitrofurantoin (> 17 = S, < 14 = R and 14–17 = I); GEN: Gentamycin (> 15 = S, < 12 = R and 12–15 = I); NAL: Nalidixic acid (> 19 = S, < 13 = R and 13–19 = I); OFL: Ofloxacin (> 13 = S, < 10 = R and 10–13 = I); AMC: Amoxicillin-clavulanate (> 20 = S, < 19 = R and 19–20 = I); TET: Tetracycline (> 19 = S, < 14 = R and 14–19 = I).

4. Discussion

Salmonella spp. and in particular the serovars, *S*. Typhimurium and *S*. Enteritidis which were specifically sought for in this investigation were detected by the traditional cultural method and PCR. Both methods were used for a very accurate confirmation of the presence of these organisms in beef and chicken. Whyte *et al*.[25] stated that the combination of both PCR test and phenotypic characterization will provide a better profile of the prevalence of *Salmonella* in the carcasses of broiler. *Salmonella* was isolated from raw beef and chicken retailed in our markets. This may be due to the unhygienic nature of our abattoirs, the dirty environment, wash water, the beef and chicken handlers who have little or no knowledge of hygiene, processing equipment which includes knives, cutlasses and basins, mode of transportation in dirty vans and in unpresentable manner to the retailing points and handling of the beef and chicken by the buyers and retailers. The two serovars, *S*. Typhimurium and *S*. Enteritidis were specifically sought for in this our investigation because they have been previously reported to be the most important serovars among the many serovars of *Salmonella* in food animals.

Salmonella spp. were more commonly isolated from raw beef samples than from chicken samples. The reason could probably be that beef is left exposed on dirty wooden tables (good source for biofilm formation) during retail. The tables are used on daily basis without washing and disinfection. Buyers handle beef with their dirty hands while negotiating the price. If it is unacceptable to them, they will leave the meat and go to another retailer and repeat the same process until they are able to get beef at an acceptable price. Beef is therefore exposed to serious mishandling by buyers. The chicken unlike the beef is usually sold as frozen, and so have low temperatures which limits the proliferation of organisms and thus limits the number and type of organisms that survive such condition. Chicken is not as exposed as beef during retail because they are usually stored in the freezers. However the freezing temperature is not maintained at times due to lack of constant electricity. The level of distribution of this pathogen remains alarming due to the fact that *Salmonella* spp. were isolated from all the samples.

The *fli*C gene (559 bp) was amplified in beef sample indicating presence of *S*. Typhimurium while the *sef*A gene (312 bp) was amplified in beef and chicken samples which shows presence of *S*. Enteritidis. Similar results have been obtained by previous workers. Jamshidi *et al*.[26] isolated and identified *Salmonella* spp. and *S*. Typhimurium from poultry carcasses. They selected the primers from the *inv*A and *fli*C genes, which are specifically used for the detection of *Salmonella* spp. and *S*. Typhimurium, respectively. For the rapid detection of *Salmonella* spp. and *S*. Typhimurium from poultry carcasses, they established the importance of multiplex-PCR and recommended that during slaughtering of poultry, strict hygiene and sanitary standards should be maintained. Oliveira *et al*.[27] reported that the use of *inv*A gene for detection of *Salmonella* and *fli*C gene for identification of *S*. Typhimuriumin multiplex – PCR assay from samples of poultry origin was 100% specific. The ERIC PCR assay of 23 *Salmonella* isolates from our present study revealed similar and divergent strains polymorphism.

All *Salmonella* spp. had multi-drug resistance to antibiotics, which may be due to the continuous usage of antibiotics in animal and poultry feeds and this is of great importance to human health. This is in agreement with results obtained by previous researchers[28,29].

Threlfall[30] stated that the occurrence of antibiotic resistant bacteria in food can be of great danger to public health because antibiotic resistance determinants may be acquired by other pathogenic bacteria which can render treatment very difficult.

The occurrence of *Salmonella* spp, *S.* Typhimurium and *S.* Enteritidis serovars in raw beef and chicken on retail in our markets and subsequently their multi-drug resistance has been established and this will serve as a useful tool in public heath strategy.

Conflict of interest statement

We declare that we have no conflict of interest.

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