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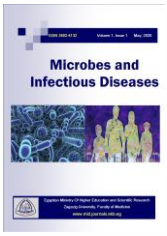

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Original article

Detection and characterization of *Salmonella enterica* from the gut and liver of *Clarias gariepinus* obtained from main fish market in Ilorin, Kwara State, Nigeria

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ABSTRACT

Background: *Salmonella* is an important foodborne pathogen prevalent in human and aquatic environments. However, identifying its source has been challenging, particularly in developing countries. This study aimed to detect the presence of *Salmonella* in fish sold at Ilorin's primary fish market in Kwara State, Nigeria. **Methods:** A total of 60 gut samples were collected from 60 *Clarias gariepinus* specimens at the major fish markets in Ilorin. Standard bacteriological procedures were employed to isolate and identify *Salmonella*. The isolates were subsequently confirmed genotypically through partial genome sequencing of the 16S rRNA gene. A phylogenetic tree was constructed using the sequences of the isolates and reference strains from the gene bank. **Results:** Among the collected samples, 11 were presumptively positive for *Salmonella*, but molecular confirmation revealed that 6 of them were indeed *Salmonella enterica*, resulting in a detection rate of 10 %. The remaining presumptive isolates were molecularly confirmed as *Lysinibacillus macrolides*, *Proteus terrae*, *Enterobacter hormaechei*, and *Alcaligenes faecalis;* most of which belong to the Enterobacteriaceae family, similar to *Salmonella*. **Conclusion:** The detection of *Salmonella* in the fish specimens sold at the market poses a substantial risk to public health. Therefore, implementing targeted interventions to control its prevalence is highly recommended.

Introduction

Fish is one of the affordable and widely acceptable sources of quality protein for human consumption [1]. It is one of the main food components of humans for many centuries and still constitutes an important part of the diet of many countries [2]. Fishes are consumed for their high biological values in terms of high protein, low level of cholesterol, and presence of essential amino acids and fatty acids. Fish also serves as an important source of income and its cultivation is a source of employment in developing countries [3]. Fish is widely acceptable sources of protein by human unlike beef or pork which are rejected by some societies either for religious or cultural reasons [4].

Clarias gariepinus is a very important freshwater fish in the Nigerian aquaculture industry [6] due to its several favorable characteristics such as the ability to tolerate a varying range of environmental conditions, fast growth rate, high fecundity rate, ease of artificial breeding and good

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market price [4,7]. Its consumption is on the increase in both rural and urban centers in Nigeria due to its highly nutritive values [4,7].

Nigeria has population estimates of over 200 million in 2021 and this population requires about 3-4 million metric tons to have their required fish protein [4]. The current fish production in Nigeria is estimated at around 1.3 million metric ton which is below half of what is required [4]. There is increased interest in aquaculture industries in Nigeria but this has been hampered by the various factors like capital, environmental contamination, diseases among others [4].

Bacteria are the most common pathogens of cultured warm water fish, implicated in the huge losses to the aquaculture industry. Many bacteria which are considered to be saprophytic are normally commensally associated with the host or live free in the environment. These bacteria can cause diseases when a fish's immune system is compromised either due to stress exposure or adverse environmental conditions. It has been reported that introduction of pollutants into aquatic bodies leads to stress that triggers numerous infectious and non-infectious diseases in the aquatic lives and pose dangers to public health [8,9]. Bacteria found in fish are generally classified into non-indigenous and indigenous groups. The non-indigenous bacteria include *Clostridium botulinum*, *Listeria monocytogenes*, *Salmonella* spp., *Shigella* spp., and *Escherichia coli*. On the other hand, indigenous bacteria include *Vibrio* species, *Staphylococcus aureus*, *Aeromonas* species and *Pseudomonas* species [10]. *Bacillus*, *Proteus*, *Pseudomonas*, *Klebsiella*, *Streptococcus*, *Salmonella*, *Staphylococcus*, *Micrococcus*, *Serratia* and *Escherichia* are found in the skin and intestine of fish [11].

Salmonella is a genus of Gram-negative non-spore forming facultative aerobic zoonotic pathogens. Fishes are among many animals that serve as reservoirs for members of this genus. Fish serves as source of *Salmonella* infection to human either directly via contact or indirectly through the consumption of contaminated products [12]. Salmonellosis has been reported to be one of the major public health challenges globally [13]. Salmonellosis due to nontyphoidal *Salmonella* causes high morbidity and mortality and great economic losses especially in the developing countries [13]. *Salmonella enterica* serovars are well documented in Nigeria in various animals and human but the report of *Salmonella* from aquatic environment is scanty [14]. *Clarias gariepinus* has been incriminated in the transmission of *Salmonella* species, *Staphylococcus* species, and *Aeromonas* species, which are the causal agent of human food-borne infection and intoxication [12]. Over 90 % of *Salmonella* infections in humans have been traced to consumption of contaminated foods of animal origin including fish [14]. High fever, gastroenteritis, bacteremia, vomiting, abdominal discomfort, and nausea are associated with human salmonellosis [13,14].

Diagnosis of bacterial infections in fish can be achieved through culture dependent or culture independent methods [4]. The culture dependent involved inoculation of samples from the diseased fish on selective agars and identification of resulting colonies using various biochemical tests. The culture independent involved isolation of DNA directly from samples or from cultured organism and identify the organism involved using molecular methods such as polymerase chain reaction or sequencing. Sequencing of the bacterial 16S rRNA gene has been used for several decades to identify clinical and environmental isolates and to assign phylogenetic relationships [5].

The potential role of fish in human *Salmonella* infections in the region remains unclear. Identifying the specific *Salmonella* genotypes associated with *Clarias gariepinus.*

Fishes specimens from local fish markets is essential for understanding this potential link. This study focuses on characterizing various *Salmonella* genotypes found in *Clarias gariepinus* sourced from the Ilorin fish market.

Materials and methods

Study area

The experimental fishes for the study were purchased from Unity live fish market, Obo-road, Ilorin, which is the main fish market in the Ilorin metropolis. The fish market is located at 8o28' 500.61" N, 4o33'400 09" E. This location was purposively selected due to its popularity, receiving fish from both cultured and wild sources in Kwara state.

Sources of fish samples

Clarias garepinus (N=60, 500g-700g average weight) were purchased from the main live fish markets in the Ilorin metropolis, Kwara State,

Nigeria. The fishes were transported within 30 minutes to Veterinary Microbiology laboratory, University of Ilorin in a lidded plastic container (50cm x 33cm x 33cm). The live, apparently healthy fish were humanely gutted and samples were aseptically collected from the intestine, stomach, and liver making a total of 180 samples.

Isolation and identification

The gut and liver sample analyses were done following standard protocols [13]. Briefly, 1 g of each sample was pre-enriched in 9 ml of buffered peptone water (Oxoid, Hampshire, UK) and incubated at 37 ℃ for 24 hours. The pre-enriched culture was later enriched in Rappaport Vassiliadis (RV) (Oxoid, Hampshire, UK) broth at the ratio of 1:9 and incubated at 42 ℃ for 24 hours (RV was used for enrichment because Selenite F is better for samples of faecal origin). A loop full of the enriched broth was sub-cultured on xylose lysine deoxycholate agar (XLD) (Oxoid, Hampshire, UK) and incubated at 37 ℃ for 24 hours (XLD was used for selective plating because of its high selectivity *Salmonella* than Salmonella-Shigella agar). Presumptive pinkish colonies with the dark center were subjected to biochemical testing including urease, citrate utilization, triple sugar iron agar, and motility tests as previously described [13]. Pure colony of each isolate was stored on Mueller Hinton broth containing 20 % glycerol at -20 ℃ for further use.

Genotyping of the isolates

Isolates prepared from the stocked pure colony was inoculated in brain heart infusion broth (BHB) (Oxoid, Hampshire, UK) and sent under the cold chain to the Bioscience laboratory at the International Institute for Tropical Agriculture (IITA), Ibadan, Nigeria for molecular analysis.

DNA extraction

DNA extraction was done according to the protocol previously described [15]. Briefly, single colony grown on nutrient agar (Oxoid, Hampshire, UK) was transferred to 1.5 ml of BHB, and cultures were grown on a shaker for 48 h at 37 ℃. After this period, cultures were centrifuged at 4600 ×g for 5 minutes. The resulting pellets were re-suspended in 520 µl of TE buffer (10 mM Tris-HCl, 1mM EDTA, pH 8.0). 15 µl of 20 % SDS and 3 µl of proteinease K (20 mg/ml) were then added. The mixture was incubated for 1 hour at 37℃, then 100 µl of 5 M NaCl and 80 µL of a 10 % CTAB solution in 0.7 M NaCl were added and vortexed. The suspension was incubated for 10 min at 65 ℃ and afterwards, kept on ice for 15 minutes. An equal volume of chloroform and iso-amyl alcohol (24:1) was added, followed by incubation on ice for 5 minutes and centrifugation at 7200 ×g for 20 minutes. The aqueous phase was transferred to a new tube and isopropanol (1:0.6) was added and DNA precipitated at –20 ℃ for 16 hours. DNA was collected by centrifugation at 13000 ×g for 10 min, washed with 500 µl of 70 % ethanol, air-dried at room temperature for approximately three hours, and finally dissolved in 50 µl of TE buffer.

Polymerase chain reaction

PCR sequencing preparation was done as previously described [16] using cocktails consisted of 10 µl of 5x GoTaq colourless reaction, 3 µl of 25 mM $MgCl_2$, 1 µl of 10 mM of the dNTPs mix, 1 µl of 10 pmol each 5'-AGAGTTTGATCM TGGCTCAG-3' and 5′-AAGGAGGTGATCCAGCC-3′ primers and 0.3 units of Taq DNA polymerase (Promega, USA) made up to 42 µl with sterile distilled water and 8µl DNA template. PCR was carried out in a GeneAmp 9700 PCR System Thermal cycler (Applied Biosystem Inc., USA) with a PCR profile consisting of an initial denaturation at 94 ℃ for 5 minutes; followed by a 30 cycles annealing consisting of 94 ℃ for 30 seconds, 50 ℃ for 60 seconds, and 72 ℃ for 1 minute 30 seconds extension, and a final termination at 72 ℃ for 10 minutes and chill at 4 ℃.

Purification of amplified product

The amplified fragments were ethanol purified to remove the PCR reagents. Briefly, 7.6 µl of Na acetate 3M and 240 µl of 95 % ethanol were added to each about 40 µl PCR amplified product in a new sterile 1.5 µl eppendorf tube, mixed thoroughly by vortexing and kept at -20 ℃ for at least 30 minutes. Centrifugation for 10 minutes at 13000 ×g and 4 ℃ followed by removal of supernatant (invert tube on trash once) after which the pellet was washed by adding 150 µl of 70 % ethanol and mix, then centrifuged for 15 min at 7500 ×g and 4℃. Again, supernatant was removed (invert tube on trash). The tube was then inverted on a tissue paper and for drying in the fume hood at room temperature for 10-15 minutes. The tube was re-suspended with 20 µl of sterile distilled water and kept in -20 ℃ before sequencing. The purified fragment was checked on a 1.5 % Agarose gel and ran on a voltage of 110V for about 1 hour. The presence and quantification of the purified product

was done using Nanodrop model 2000 (Thermo Scientific, USA).

Sequencing

The amplified fragments were sequenced as previously described [17] using a genetic analyzer 3130 xL sequencer (Applied Biosystems, Inc., USA) according manufacturers' manual using BigDye terminator v3.1 cycle sequencing kit.

Phylogenetic analysis

Bio-Edit software version 7.7.1 was used to generate consensus sequence from the Fastq data and the sequences were blasted on NCBI data base to identify the isolates. The sequences were deposited in gene bank for accession numbers and the sequences were aligned using Aliview version 1.28. The Maximum Likelihood and Tamura-Nei model phylogenetic tree was constructed using MEGA version X as previously described [18].

The sequences of the isolates deposited in NCBI database are available on https://www.ncbi.nlm.nih.gov/nuccore/?term=isolation+and+Molecular+identification+of+Salmonella+spp+in+the+gut+of+Clarias+gariepinus.

Results

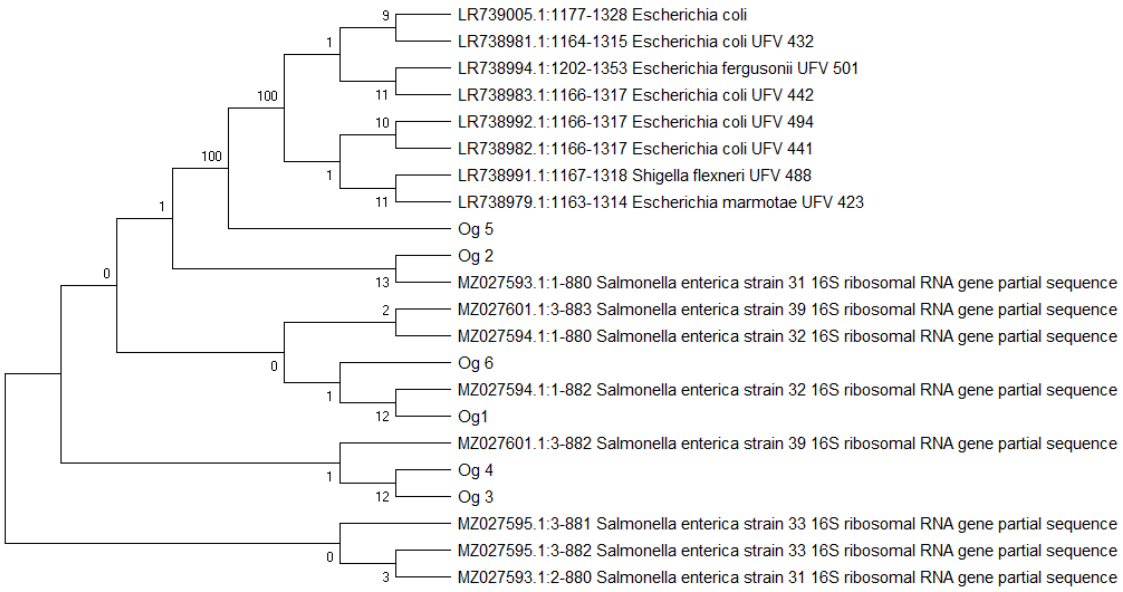
Out of the sixty fish samples, six *Salmonella* were recovered from the different parts of their gut and liver given the detection rate at 10.0 %. Even though, eleven of the isolates were presumptively identified as *Salmonella* with biochemical testing.

The remaining five presumptive isolates were molecularly confirmed as *Lysinibacillus macrolides*, *Proteus terrae*, *Enterobacter hormaechei*, and *Alcaligenes faecalis* which are majorly in the same enterobacteriaceae family as *Salmonella*. The isolates were mostly detected in the intestine (n=5) while only one isolate was detected from the esophagus **(Table 1)**. **Figure (1)** shows the phylogenetic analysis of the isolates that were molecularly confirmed as *Salmonella* and the reference strains of *Salmonella* from NCBI data base. Reference strains of *Escherichia coli* were used for the outgroup. Each of the *Salmonella* isolates clustered with distinct reference *Salmonella* strain. Og1 clustered with *Salmonella enterica* strains 32 and 39 with accession numbers MZ027594.1:1-882 and MZ027601.1:3-882 respectively. Og2 clustered distinctly with the *Salmonella enterica* reference strain 31, 39, and 32 with accession numbers MZ027593.1:1-880, MZ027601.1:3-883, and MZ027594.1:1-880 respectively. Similarly, the isolates Og3 and Og4 associated distinctly with *Salmonella enterica* strains 39 and 33 with accession numbers MZ027601.1:3-882 and MZ027595.1:3-881 while the isolate Og6 clustered distinctly with the *Salmonella enterica* reference strain 32 with accession number MZ027594.1:1-880. All the isolates dissociated themselves distinctly from the outgroup (*Escherichia coli*).

Table 1. Genotypic and phenotypic characteristics of *Salmonella enterica* from gut and liver *of Clarias gariepinus* from the fish market in Ilorin, Kwara State, Nigeria

S/N	Sample source	Biochemical characteristics				Presumptive Identification	Molecular Characteristics			
							Species	Q. C (%)	Per. Ident (%)	Accession
		URE	H_2S	CAT	CIT					
1	Oesophagus	-	+	+	+	*Salmonella*	*Salmonella enterica*	99	99.43	MZ027593
2	Liver	-	+	+	+	*Salmonella*	*Salmonella enterica*	99	99.32	MZ027594
3	Intestine	-	+	+	+	*Salmonella*	*Salmonella enterica*	100	99.32	MZ027595
4	Stomach	-	+	+	+	*Salmonella*	*Salmonella enterica*	96	99.45	MZ027601
5	Intestine	-	+	+	+	*Salmonella*	*Salmonella enterica*	99	98.81	MZ027597
6	Liver	-	+	+	+	*Salmonella*	*Salmonella enterica*	100	100.00	MZ027593
7	Liver	-	+	+	+	*Salmonella*	*Lysinibacillus macroides*	91	99.45	MZ027596
8	Intestine	-	+	+	+	*Salmonella*	*Proteus terrae*	100	100.00	MZ027598
9	Liver	-	+	+	+	*Salmonella*	*Enterobacter hormaechei*	100	100.00	MZ027599
10	Intestine	-	+	+	+	*Salmonella*	*Alcaligenes faecalis*	99	96.81	MZ027600
11	Intestine	-	+	+	+	*Salmonella*	*Alcaligenes faecalis*	99	96.81	MZ027602

Figure 1. The phylogenetic tree of the *Salmonella* isolates from *Clarias gariepinus* in Ilorin, Nigeria and the reference strains of *Salmonella enterica* from NCBI and reference strain of *Escherichia coli* (outgroup) based on 1000 bootstrap values



Discussion

Salmonellosis is a one of the global zoonotic pathogens whose occurrence in animals poses serious threat to public health. In this study, we have conducted a molecular analysis of *Salmonella* isolates from fish sold at the main fish market in Ilorin, Kwara State, using a 16S rRNA sequence to detect the distribution of *Salmonella* in the fish sold to end-consumers. The occurrence of *Salmonella* from fish in the present study is in agreement with previous study in Nigeria [14] but higher rate (16.7 %) was reported in Kenya [19]. The differences may be due to differences in geographical location, period of study and sensitivity of the methods used in the detection of the organism. The fact that some isolates that were identified biochemically as *Salmonella* were confirmed molecularly to be other organisms reaffirm the sensitivity of the genotypic over the phenotypic methods in the identification of the bacteria [5]. The presence of *Salmonella* in fish has been reported as one of the major routes for the transmission of *Salmonella* to humans [14, 20]. *Salmonella* has been reported to be a normal contaminant in fish, being introduced via water contaminated with the pathogen or during handling [21]. The presence of this pathogen in fish could be due to pollution of the water supply to the ponds by storm water or runoffs that contain deposits from animals, rural and urban settlements, and agriculture. This may pose a high risk to public health [22] since the fish are consumed by humans. Many predisposing factors such as season, contact with fish, eating habits, and immune status, have been identified to determine the nature and severity of the disease caused by pathogens transmitted from fish to humans [14]. Handling, a multistep process that begins from harvest through transportation and ends in the fish market, is another possibility for contamination of fish sold for human consumption with *Salmonella* as previously reported [20]. Handling could also be a *Salmonella* transmission factor as it is reported by [23] that during the handling of fish, dangers of *Salmonella* transmission might arise from catching, slaughtering, and processing. In the current study, the *Salmonella* contamination of fish sold in the fish market could also have arisen from sources such as unorthodox use of animal feces as fertilizers on farmland located near the rivers that serve as source of water for fish ponds, this go in line with [24] who observed that detection (using gene specific PCR) of invasive *Salmonella* genes from fish and fish products is known to be source of environmental contamination and human infection. Accordingly, the topsoil is washed away to the river during the rainy season resulting in contamination of water bodies. A survey of water bodies in this regard would be necessary for

future study. Another factor is improper sewage disposal leading to environmental contamination with human feces since pollution has been adjudged to play important roles in the dissemination bacterial pathogens to both fish and humans [8]. Aquatic pollution was reported to be one of the major setbacks affecting aquaculture production and public health especially in the developing countries where little or no emphasis is applied on environmental sanitation policies [8]. Transportation of fish in dirty containers and vehicles to fish market might also be contributory to the transmission and spread of the pathogens to the fish sold for human consumption at markets [25]. Ilorin is now one of the major hubs for catfish production in Nigeria and West Africa, hence, the detection of *Salmonella* in fish sold for human consumption in the Ilorin metropolis is of high public health concern.

Conclusion

The detection of *Salmonella* genotypes in the fish sold at fish markets in the Ilorin metropolis indicated the possibility of contamination of water sources for aquaculture in the study area. Since infected/contaminated fish can serve as a source of human infections; it is therefore important that fish from the study area are properly cooked before consumption. Strict fish quality control measures at fish markets are also advised. However, there is the need for further study on the occurrence and spread of *Salmonella* species in the study area, with a larger sample size covering more live fish markets in the state. Such study should involve sampling of the fish in wild and cultured environments, and contact persons along the production and distribution value chains. The study should also determine the serovars and their susceptibility patterns.

Conflict of interest

None

Author contribution

Conceptualization and study design was done by YOY, OOO and AOA. Data collection analysis and interpretation was done by YOY, AOA, WAJ and OAA. Drafting of the article and revising it critically for important intellectual content was carried out by YOY, OOO, FJF and IAR. All authors approved the final copy meant for submission.

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