RESEARCH ARTICLE

Sokoto Journal of Veterinary Sciences

(P-ISSN 1595-093X: E-ISSN 2315-6201)

http://dx.doi.org/10.4314/sokjvs.v16i2.10

Kia *et al*./*Sokoto Journal of Veterinary Sciences*, **16**(2): 75 - 82.

Occurrence of Salmonella and Shigella in edible frogs (*Hoplobatrachus* spp) from Hanwa Frog market Zaria, Nigeria

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Abstract

Frogs have been associated with bacterial infection among those who handle them resulting in symptoms such as diarrhoea, abdominal cramps, fever and vomiting. Frogs are a rich source of proteins and they are considered a delicacy by some in Nigeria. Considering the high demand for edible frogs, it is important to determine the occurrence of Salmonella and Shigella organisms from edible frogs (*Hoplobatrachus* spp). Edible frogs (n=202) were collected from February to July, 2016, from the Hanwa frog market, Zaria, Kaduna State. The intestinal contents of each sampled frog were scraped into the selenite broth bottles and cultured on Deoxycholate Citrate Agar for enrichment and isolation respectively. Biochemical test and sugar fermentation tests were carried out on the suspected isolates. Overall, twenty seven 27(13.37%) of the processed samples were suggestive of *Shigella,* while 22(10.9%) were suspect Salmonella organisms. There was no significant association between sex of the frogs and the isolation of Shigella and Salmonella organisms, despite the high occurrence of Shigella organism (14.17%) in the males. Source wise the occurrence of Salmonella in frogs was high in Tudun Wada (20%), while Katsina (8.5%) had the least. There was also no association between source and Shigella organisms. Frogs within the weight range of 175-224g had the highest occurrence rate for Shigella isolation, while frogs of 73-125g weight range had the highest occurrence rate for Salmonella isolation. This study shows the presence of Shigella and Salmonella organisms in the intestinal contents of frogs. Therefore the unhygienic and unsanitary environment, handling and processing of frogs is of great public health concern and as such measures are to be put together to ensure safety and wholesomeness of the frog meat been sold for human consumption.

Publication History:
Received: 04-10-2017
Accepted: 22-02-2018

Keywords: Edible, Frog, Safety, Salmonella , Shigella, Zaria

Introduction

Frogs belong to a diverse and largely carnivorous group of short bodied tailless amphibians composing the order Anura (Singh, 2017). Frogs can come into direct contact with various bacteria constantly, which may be present in the soil, water body or present in the arthropods that they eat (Rebollar *et al*., 2016). Frogs may be predisposed to bacterial infection when they are stressed. Pathogens build up in enclosed environments where frogs can be found and can cause infection (PetMD, 2008). The bacterial organisms commonly affecting frogs predilect in their gastrointestinal tract, thus upon consumption by man would result in infection by the presence of bacteria toxin (Marler, 2009).

Salmonella is a rod shaped gram negative bacteria of the family *Enterobacteriaceae*. *Salmonella* species are facultative intracellular pathogens, many infections are due to the ingestion of contaminated food (Ryan & Ray, 2004). Frogs have been reported to serve as reservoir host of Salmonella organisms; pathogenic strains of Salmonella may be transmitted from frogs to human by the faecal-oral route (Hoelzer *et al*., 2011). *Salmonella anatum, Salmonella poona and Salmonella richmond* from frogs seem important in the epidemiology of Salmonella infections because of the association between these serotypes with man and domestic animals (Leeuwenhoek *et al*., 1974).

The actual number of the human population in Nigeria has doubled during the past 20 years and is need for animal protein consumption has also increased, thereby resulting in increased, consumption of frogs; therefore, the greater the number of the populace consuming it, the greater the infectivity rate in cases of unhygienic preparation methods (CDC, 2008).

Shigella is a genus of Gram-negative, facultative anaerobic, non-spore-forming, non-motile, rod-shaped bacteria closely related to Salmonella . The genus is named after Kiyoshi Shiga, who first discovered it in 1897 (Yabuuchi, 2002). Aquatic bodies have been reported to be contaminated with human faeces and this can expose the water bodies to Shigella organisms. This may result in infection of frogs leading to decline in their population. When frog meat is undercooked, it may result in zoonotic disease in humans. Human cases of Shigella are typically acquired through contact with infected persons or ingestion of food contaminated with Shigella bacteria (Gupta *et al*., 2004).

The consumption of frog in many part of the world and in Nigeria in particular, is on the increase. (Stuart *et al.,* 2004). In Nigeria, frogs are collected on a local scale for human consumption as an essential source of animal protein. There is little or no work done on the isolation of *Shigella* spp and *Salmonella* spp from intestinal contents of edible frogs in Zaria Kaduna State, Nigeria. Hence, the need for this research.

Materials and Methods

Study area

The study area is Hanwa frog market, located in Zaria Local Government Area of Kaduna State. It is about 141 mi (or 227 km) North of Abuja, Nigeria`s Federal capital city. The area located at latitude $11^{o}04'$N and longitude $7^{o}42'$E. Edible frogs (*Hoplobatrachus* spp) used for this study were sourced from different areas in Kaduna and neighbouring states and brought to Hanwa frog market which serves as collection, slaughter, processing and distribution point (Kia *et al*., 2017).

Sample collection

The frogs were collected from the Hanwa frog market from February to July, 2016. Twelve batches from which a total 202 were collected and labelled appropriately. They were grouped according to their source. The sources of the frogs brought from to Hanwa market included;

i. Zaria metropolis (Tudun Wada n=20, Sabon Gari n=20, University Farm n=27, Dabai n=20, Kafuru n=10, Muchia n=10)
ii. Kaduna (Maraban Jos n=10)
iii. Katsina n=35
iv. Zamfara n=15
v. Kano n=10,)

All frogs obtained from these locations were brought to the Hanwa frog market in Zaria which serves as the collection, processing and distribution center for frogs. A total of 12 batches of frogs comprising of 202 frogs in total were used for this study.

Media preparation

Enrichment was done with Selenite broth (Oxoid Limited, England). All the media used for bacterial culture and identification were prepared in the laboratory according to the manufacturers' instructions and include; Deoxycholate agar; (Oxoid Limited, England), Nutrient agar slants (Oxoid Limited, England), Triple Sugar Iron agar (Antec Diagnostic Products TMUK), Urease Agar (Oxoid LTD, London), Simmon's citrate Agar (Himedia TM), Nutrient Agar (Oxoid LTD, London), Sulphur Indole motility (SIM) agar, Methyl Red/ Voges Proskauer agar and sugars (Arabinose, Maltose, Mannitol, Sorbitol, Dulcitol and Lactose) .

Sample processing

The frogs were placed inside a polythene bag and inserted into a refrigerator to achieve anaesthesia after which they were weighed, dissected and their sexes were determined as described elsewhere (Kia *et al*., 2017). Out of the 202 frogs that were sampled, 127 were males and 75 were females. The intestinal contents were scraped and inoculated each into 5ml of prepared Selenite F broth in 10ml capped tubes and incubated at 37^{o}C for 24 hours.

Bacterial isolation and identification

A loopful of each broth inoculum with identifiable growth on Selenite broth was streaked on

Deoxycholate Citrate Agar (DCA) and incubated for 24 hours at 37°C. The colonial morphological growth of Salmonella and Shigella as observed on DCA was a smooth, raised, shiny or transparent colony with or without black centers was considered to be Salmonella and Shigella suspect, respectively (Cheesbrough, 1984; Hunt & Goldsmid, 1990). The cultured organism was inoculated and stored on a Nutrient agar slant and refrigerated. Standard biochemical tests were used to identify Salmonella and Shigella among suspect isolates using conventional methods. Test conducted include; Triple sugar iron (TSI) prepared according to the manufacturer's recommendation. The isolate inoculated on TSI by stabbing. This was incubated for 37°C for 24hours. Isolate were also streaked on prepared Urease agar slants, incubated at 37°C for 24 hours. Subsequently, Sulphur Indole motility (SIM) agar was inoculated for motility, H_2S and indole production. Presence of cloudiness around the stab-line after 24 hours at 37°C incubation indicated motile organism. Following addition of 2 drops of Kovac's reagent to the SIM tubes, a pinkish-red colour layer indicates indole production. Blackening of the stab line shows H_2S. Other biochemical tests, viz; MR, VP, Indole and Citrate were also conducted by inoculating putative isolates as described by Cheesbrough (1984) and Gaurav *et al*. (2013).

Further confirmation was carried out using sugar fermentation test following the recommendation of (Cheesbrough, 1984). A 10% solution of Lactose, Dulcitol, Sorbitol, Maltose, Mannitol and Arabinose, each was prepared and autoclaved at 115°C for 20 minutes. Approximately 0.1g of Bromothymol blue was added to 2.5mls 0.1 molar NaOH and dissolved in 47.5mls of distilled water. The mixture was then added to a salinized buffered peptone water and autoclaved at 115ºC for 20 minutes. To 9mls of this solution, 5mls of the sugar solution was added and thoroughly mixed. Into each test tube 5mls of the mixture was pipetted and suspected Salmonella or Shigella isolates as the case may be were inoculated into the solution and incubated at 37°C for 24 hours. Colour change of sugars indicated fermentation (Cheesbrough, 1984).

Data analyses

The data obtained were analyzed using statistical package for social science 16th edition.

Results

The results were represented in tables and percentages. Chi-square was used to test for association between sex, location and *Shigella* or *Salmonella* spp isolated from the intestinal content of frogs sold for human consumption in Hanwa Zaria, Kaduna State. Values of $p < 0.05$ were considered significant.

Salmonella *and* Shigella were found in 22 (10.9%) and 27 (13.4%) of the 202 frogs sampled. Of these 202 frogs sampled 127(62.9%) were males and 75 (37.1%) were females (Tables 1 and 2).

The samples were evaluated for statistical relationship between the sex of the frogs and the Salmonella *and* Shigella occurrence rate (Tables 1 and 2). Among the frogs which had Salmonella (n=22) and Shigella (n=27), males were more infected with Salmonella (72.8%) and Shigella (66.7%) than the females (27.2% and 33.3% respectively), thus giving a higher male to female ratio for both Salmonella and Shigella occurrence. This relationship was not statistically significant as the *p* values were both > 0.05 (Tables 1 and 2).

Table 1: Sex and weight distribution of edible frogs (*Hoplobatrachus* spp) infected with Salmonella from Hanwa frog market Zaria, Kaduna State, Nigeria

Variable	Total Number Sampled (%)	No. positive (Source Specific Rate (%)	Proportional positivity Rate (%)	x^2	P-Value
Sex					
Male	127(62.9)	16(12.5)	72.8	1.027	0.35
Female	75 (37.1)	6(8)	27.2		
Weight (grams)					
20- 72	40(19.8)	4(10)	18.2		
73-125	130(64.4)	14(10)	63.6		
126-178	23(11.4)	4(17.4)	18.2		
179-231	6(3)	0(0)	0		
232-284	3(1.5)	0(0)	0		
Total	202	22(10.9)	100		

Table 2: Sex and weight distribution of edible frogs (*Hoplobatrachus* spp) infected with Shigella from Hanwa frog market Zaria, Kaduna State, Nigeria

Variable	Total Number Sampled (%)	No. positive (Source Specific Rate (%)	Proportional Positivity Rate (%)	x^2	P-Value
Sex					
Male	127(62.9)	18(14.2)	66.7	0.1923	0.661
Female	75 (37.1)	9(12)	33.3		
Weight (grams)					
20- 72	40(19.8)	1(2.5)	3.7		
73-125	130(64.4)	16(12.3)	59.3		
126-178	23(11.4)	5(21.7)	18.5		
179-231	6(3)	4(66.7)	14.8		
232-284	3(1.5)	1(33.3)	3.7		
Total	202	27(13.4)	100		

Table 3: Locational distribution of edible frogs (*Hoplobatrachus* spp) infected with Salmonella from Hanwa frog market Zaria, Kaduna State, Nigeria

S/N	Location/Code	Total Number Sampled	No. positive (source specific rate (%)	Proportional positivity rate (%)
1	Tudun wada / TW	20	4(20)	18.2
2	Sabon gari/ SG	20	3(15)	13.6
3	University farm/UF	27	3(11.1)	13.6
4	Katsina/KT	35	3(8.5)	13.6
5	Zamfara/ZF	15	2(13.3)	9.1
6	Maraban jos/MT	10	3(20)	13.6
7	Muchia/MCH	10	3(30)	13.6
8	Giwa/GW	10	0(0)	0
9	Wucheri/WCH	15	2(13.3)	9.1
10	Kano/KN	10	0(0)	0
11	Dabai/DAB	20	0(0)	0
12	Kafuru/KF	10	0(0)	0
	Total	202	22(10.9)	100

The samples were also evaluated for relationship in weight and the occurrence of Salmonella *and* Shigella. Findings revealed the highest occurrence (17.4%) among the weight range 126g-178g for Salmonella and 66.7% for Shigella of weight range 179g-231g, while the weight specific rate showed that 63.6 and 59.3% of frogs all within the weight range of 73-125g were found with Salmonella *and* Shigella, respectively (Tables 1 and 2).

The occurrence of Salmonella *and* Shigella were also evaluated based on the source location (Tables 3 and 4) and it revealed the highest occurrence of Salmonella *and* Shigella in frogs sourced from Muchia (30%) and Tundun Wada (40%), respectively. Bio-typing for Salmonella and Shigella resulted in 22 and 30 suspected isolates respectively (Tables 5 and 6). Further analysis using Sugar fermentation tests for Salmonella and Shigella yielded 22 and 27 of the isolates gave the expected outcome (Tables 7 and 8). From the results (Tables 7 and 8), typical Shigella suspect gave alkaline and acid reaction on the slant and butt of the Triple Sugar Iron. There was no H_2S and indole production. Absence of cloudiness around the stab-line after 24 hours at 37^oC incubation indicated no motile organisms.

Discussion

Salmonella and Shigella presence in 10.9% and 13.4%, respectively, of frogs sampled in this study may be indicative of water contamination since frogs are not natural host nor reservoirs of *Shigella* spp (Pond, 2005). However, frogs serve as reservoir host of the Salmonella organism (CDC, 2008).

Table 4: Locational distribution of Shigella isolated from edible frogs sold in Hanwa Zaria, Kaduna State Nigeria

S/N	Source/Codes	Total No. Sampled	No. Positive (%)
1.	Tudunwada (TW)	20	8(40.0)
2.	Sabongari(SG)	20	5(25.0)
3.	Zamfara(ZF)	15	1(6.7)
4.	Katstina(KT)	35	3(8.6)
5.	Kano (KN)	10	0(0.0)
6.	University farm (UF)	27	2(7.4)
7.	Wucheri (WCH)	15	2(13.3)
8.	Muchia (MCA)	10	2(20.0)
9.	Dabai (DA)	20	2(10.0)
10.	Giwa (GW)	10	1(10.0)
11.	Kafuru (KF)	10	1(10.0)
12.	Maraban Jos (MR)	10	0(10.0)
	Total	202	27(13.4%)

Table 5: Biochemical Test for the suspected Salmonella isolate in Edible Frogs (*Hoplobatrachus* spp) from Hanwa Frog Market Zaria, Kaduna State, Nigeria

Location code (sample Id)	T.S.I	Urease	Citrate	Indole	MTY	MR	VP	Salmonella
U.F (3, 10, 12)	Alk/Acid $_+$ H_2S	-VE	+VE	-VE	+VE	+VE	-VE	✓
KT (4, 5, 9)	Alk/Acid $_+$ H_2S	-VE	+VE	-VE	+VE	+VE	-VE	✓
ZF (2, 6)	Alk/Acid $_+$ H_2S	-VE	+VE	-VE	+VE	+VE	-VE	✓
MR (4, 6)	Alk/Acid $_+$ H_2S	-VE	+VE	-VE	+VE	+VE	-VE	✓
MCA (7, 8, 10)	Alk/Acid $_+$ H_2S	-VE	+VE	-VE	+VE	+VE	-VE	✓
WCH (7, 12)	Alk/Acid $_+$ H_2S	-VE	+VE	-VE	+VE	+VE	-VE	✓
TW (6, 7, 15, 16)	Alk/Acid $_+$ H_2S	-VE	+VE	-VE	+VE	+VE	-VE	✓
SG (2, 6, 16)	Alk/Acid $_+$ H_2S	-VE	+VE	-VE	+VE	+VE	-VE	✓

KEYS

UF= University Farm; KT= Katsina; ZF= Zamfara; MR= Maraban Jos; MCA= Muchia; WCH=Wucheri; TW=Tudun wada; SG=Sabon gari; Alk= Alkaline; T.S.I=Triple sugar iron; MTY=Motility; MR=Methyl Red; VP=Voges Proskauer

These frogs may serve as a means of transmission of zoonotic diseases both to man and animals especially to individuals processing them without good hand washing personal hygiene practice. Consumption of improperly cooked infected frogs may serve as a route of transmission of Salmonella and or Shigella (Mazzoni *et al*., 2003). This implies that the water bodies in which the frogs were sourced from may be contaminated by these infectious organisms or they might have acquired them from their feed. As such individuals involved in the catching or hunting and processing these frogs for meat purpose should ensure a high level of good hygienic practices as well in order to prevent and reduce the rate of infection.

Varying percentages of Salmonella (for example, Tudun Wada (20%), Sabon gari (15%), Wucheri (13.3%), Katsina with 8.5%) were found in the various areas sampled. Therefore this implies that the various locations may probably be contaminated with the salmonella microbes. The result of this study shows that the frogs processed for human consumption could be a source of bacterial infection to man and animals

Table 6: Biochemical test result of the Shigella Isolate in edible Frogs from Hanwa Frog Market Zaria, Kaduna State, Nigeria

Location code (sample Id)	TSI	Urea	Citrate	Motility	Indole	H_2S	MR	VP	Shigella *suspect*
TW (2, 4, 7, 9, 10, 11, 15, 16)	ALK ACID	-	-	-	-	-	+	-	✓
SG (8, 9, 12, 14, 18)	ALK ACID	-	-	-	-	-	+	-	✓
UF (7, 14, 19)	ALK ACID	-	-	-	-	-	+	-	✓
KT (5, 9, 10)	ALK ACID	-	-	-	-	-	+	-	✓
ZF (8, 10)	ALK ACID	-	-	-	-	-	+	-	✓
WC (5, 11)	ALK ACID	-	-	-	-	-	+	-	✓
MC (4, 8)	ALK ACID	-	-	-	-	-	+	-	✓
DA (7, 16)	ALK ACID	-	-	-	-	-	+	-	✓
KF (4, 13)	ALK ACID	-	-	-	-	-	+	-	✓
GW (8)	ALK ACID	-	-	-	-	-	+	-	✓

Keys: UF= University Farm; KT= Katsina; ZF= Zamfara; MR= Maraban Jos; MCA= Muchia; WCH=Wucheri; TW=Tudun wada; SG=Sabon gari; GW= Giwa; Alk= Alkaline; T.S.I=Triple sugar iron; MTY=Motility; MR=Methyl Red; VP=Voges Proskauer

Table 7: Sugar Fermentation Test for Suspected Salmonella Isolates in Frogs from Hanwa Frog Market in Zaria, Kaduna State, Nigeria

Location code (sample Id)	Arabinose	Dulcitol	Maltose	Manitose	Lactose	Sucrose	Sal. Susp
U.F (3, 10, 12)	+	_	+	+	_	_	✓
KT (4, 5, 9)	+	_	+	+	_	_	✓
ZF (2, 6)	+	_	+	+	_	_	✓
MR (4, 6)	+	_	+	+	_	_	✓
MCA (7, 8, 10)	+	_	+	+	_	_	✓
WCH (7, 12)	+	_	+	+	_	_	✓
TW (6, 7, 15, 16)	+	_	+	+	_	_	✓
SG (2, 6, 16)	+	_	+	+	_	_	✓

KEYS UF= University Farm; KT= Katsina; ZF= Zamfara; MR= Maraban Jos; MCA= Muchia; WCH=Wucheri; TW=Tudun wada; SG=Sabon gari; Sal Susp= Salmonella suspect

and therefore good hygienic measures should be upheld during capture and processing.

There is also the need to educate the public on the cautious use of water from such rivers or dams, also that aquatic animal from such water bodies should be properly cooked before consumption. Similarly, the presence of Shigella (13.4%) from frogs sampled in this study implies that the aquatic environment these frogs are harvested from may possess these infectious organisms. Irrigation farming among farmers in these

Table 8: Sugar Fermentation test for suspected Shigella isolates in frogs from Hanwa Frog market in Zaria, Kaduna State, Nigeria

Location Code (Sample Id)	Arabinose	Maltose	Manitole	Sorbitole	Dulcitole	Lactose	Inference
Tw (2, 4, 7, 9, 10, 11, 15, 16)	+	+	+	+	-	-	*Shigella Spp*
Sg (8, 9, 12, 14, 18)	+	+	+	+	-	-	*Shigella Spp*
Uf (14, 19)	+	+	+	+	-	-	*Shigella Spp*
Kt (5, 9, 10)	+	+	+	+	-	-	*Shigella Spp*
Zf (10)	+	+	+	+	-	-	*Shigella Spp*
Wc (5, 11)	+	+	+	+	-	-	*Shigella Spp*
Mc (4, 8)	+	+	+	+	-	-	*Shigella Spp*
Da (7, 16)	+	+	+	+	-	-	*Shigella Spp*
Kf (13)	+	+	+	+	-	-	*Shigella Spp*
Gw (8)	+	+	+	+	-	-	*Shigella Spp*

KEYS: UF= University Farm; KT= Katsina; ZF= Zamfara; MR= Maraban Jos; MCA= Muchia; WCH=Wucheri; TW=Tudun wada; SG=Sabon gari

villages is done utilizing the water from these same water bodies, hence may serve as source of contamination of vegetables and other farm produce that are not cooked prior to eating. Shigella distribution on the bases of weight showed little or no presence of the microorganism at weight below 72g and also weight above 232g and higher specific rate of 4(66.6%) was obtained in frogs between 79-231g, this may relate to young and old frogs respectively which may be deterrent on the extent of movement hence not as exposed to contaminants as the others.

There was varying degree of Shigella isolation from edible frogs sold for human consumption in Hanwa Zaria, Kaduna state. This implies that infection of edible frogs by Shigella organism and contamination of the aquatic habitats varies from one place to another. This may be dependent on the presence or absence of faecal materials, environmental contamination and surface run-off (Esona *et al*., 2010).

There should be adequate and sustained public health enlightenment on dangers associated with unhygienic processing and consumption of improperly cooked frog meat. Further studies should be carried out using serotyping and molecular techniques to identify species of Salmonella and Shigella isolates obtained from culture and biotyping.

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