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VIRULENCE GENES DETECTION AND ANTIBIOTIC RESISTANT *SALMONELLA* IN RAW AND READY-TO-EAT SNAILS (*ARACHATINA MARGINATA*) SOLD IN SELECTED MARKETS IN PORT HARCOURT

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ABSTRACT

This study investigated the presence of virulence genes and antibiotic resistant Salmonella spp. in raw and ready-to-eat snails (Archachatina marginata) vended in selected markets within Port Harcourt, Rivers State, Nigeria. Proximate composition, isolation, identification and presence of virulent genes were done using standard methods. Raw snails from Choba had Salmonella counts ranging from 3.32 to 5.04 log_{10}CFU/g. Salmonella was not detected in ready-to-eat samples from Choba. Raw snails from Rumuokoro had Salmonella counts ranging from 4.04 to 6.04 log_{10}CFU/g while three of the ten ready-to-eat samples had counts ranging from 3.53 to 3.63 log_{10}CFU/g. Raw snails from Oyigbo had Salmonella counts ranging from 4.71 to 6.67 log_{10}CFU/g with two of the five ready-to-eat samples having Salmonella counts of 3.69 and 3.51 log_{10}CFU/g. Antimicrobial susceptibility test results showed that all the isolates were resistant to augmentin, cefuroxi and cetazidime. Ten Salmonella representing 5% possessed the antibiotics resistance genes, fliC and invA, but not sefA. The presence of Salmonella in some of the ready-to-eat samples makes it objectionable for human consumption. But more worrisome is that some possess fliC and invA genes and resistant to common antibiotics used for their management. Therefore, proper processing and maintenance of quality of processed snail meat is very essential for public health safety.

Keywords: *Salmonella*, antibiotic resistance, *fliC* gene, *invA* gene, snail

INTRODUCTION

Land snail meat consumed widely serves as a good source of protein, iron, calcium, phosphorus and essential fatty acids (linoleic and linolenic (Akinnusi, 2002; Nyoagbe et al., 2016). Over the years, most farmers have carved out their niche of farming snails due to its high demand by the ever-growing population in the most populous nation in Africa. It is understood that most animals are comfortable habitats to disease causing microorganisms and possible transmission of a lot of virulent microbes to man. Snails due to their feeding habits function as carriers of several microorganisms (Adebayo-Tayo et al., 2012; Ogbonna and Inana, 2018).

Snails as molluscs have been reportedly drawn in as vector for spread of human

disease pathogens such as *Salmonella* species (Adagbada et al., 2011). *Salmonella* causes enteric fever, bacteremia, gastroenteritis, and other extra intestinal anomalies, including entering a chronic carrier state (Sheorey and Darby, 2008). Infections caused by *Salmonella* remains a key problem of public health worldwide, resulting in economic challenges in both underdeveloped and industrialized nations because of the cost of its surveillance, treatment, prevention and control (Crump et al., 2004).

The presence of enteric bacteria (especially *Salmonella* and *Escherichia coli*) in snails put them on the radar as potential medium through which diseases caused by them can be spread to humans and hence, the necessity for public consciousness on the potential community health diseases which may be associated with eating inadequately cooked snail meat.

The incidence of *Salmonella* strains that are resistant to antibiotics is a serious public health problem globally (Chiu et al., 2002). Since the first occurrence of *Salmonella* resistant to antibiotic (chloramphenicol), was reported, the rate of occurrence of *Salmonella* strains having resistance to one or more antibiotics has increased in many nations of the world (Montville and Matthews, 2008; Yoke-Kqueen et al., 2008). Antibiotics like penicillin, ampicillin, chloramphenicol and trimethoprim–sulfamethoxazole are all first line drugs for management of salmonellosis. *Salmonella* spp. resistant towards these drugs or antibiotics are said to be multi-drug resistant (MDR). The two continents with the highest prevalence of MDR *Salmonella* phenotypes are Asia and Africa (Chuang et al., 2009).

This study is aimed at investigating the presence of virulent and antibiotic resistant *Salmonella* in raw and ready-to-eat land snails (*Archachatina marginata*) vended in selected markets within Port Harcourt.

MATERIALS AND METHODS

Sample collection

Twenty-five (25) samples each of both raw and ready-to-eat snails were sourced randomly from different vendors in Choba market, Rumuokoro market, and Oyigbo Markets. They were transported in a sterilized bag to the Microbiology laboratory for analysis.

Proximate Analysis

Proximate analysis was done on the raw and ready to eat snail meat to determine their nutritional status. Determination of moisture**,** crude protein, fat, ash, total available carbohydrate and crude fibre was according to AOAC (2005) methods.

Isolation of Bacteria

Commercially available nutrient media, namely: Nutrient and *Salmonella-Shigella* agar were used for isolation. Ten grams (10 g) of the sample from different locations were added into 90 ml of peptone broth broth and homogenized using a stomacher blender for 2 min after which a ten-fold serial dilution. From the prepared dilutions, 0.1 ml of each of 10^{-4} and 10^{-5} dilutions were transferred into sterile Petri plates containing the different media used and was spread gently using sterile glass rod. The plates were incubated at ambient temperature (29±2^{o}C) for 18-24 h. Microbial count were expressed as colony forming unit (CFU/g).

Characterization and identification of Isolates

The characteristic *Salmonella* isolates on *Salmonella-Shigella* agar were further confirmed on the bases of the physiological and biochemical characteristic [Gram staining reaction, motility and biochemical tests including indole, catalase, methyl red, Voges-Proskauer, citrate and triple sugar iron agar test (TSIA) and sugar fermentation tests] (Cheesebrough, 2005).

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Antibiotics Susceptibility Testing

Antibiotic sensitivity patterns of all the confirmed isolates were performed by standard disk diffusion method according to Kirby-Bauer on Mueller-Hinton agar (Titan, Biotech Ltd, Indian) following the procedures recommended by Clinical and Laboratory Standard Institute (CLSI) as employed by Eruteya & Osariemen (2021). Eight commonly used antibiotics (µg/disc) viz. amoxicillin-clavulanate or augmentin (AUG), gentamycin (GEN), nitrofurantoin (NIT), cefuroxime (CRX), ofloxacin (OFL), cefixime (CXM), ciprofloxacin (CPR), cetazidime (CAZ), Abtek, (UK) were tested. From an overnight culture *Salmonella* spp., 0.5 MacFarland turbidity standards was prepared in sterile saline, from which 0.1ml was inoculated onto Mueller Hinton agar. Thereafter, antibiotic discs were carefully and aseptically placed on the surface of the agar. The plates were incubated at ambient temperature (29±2^{o}C) 24h. Zone of inhibition was measured in millimeter.

MOLECULAR ANALYSIS

DNA Extraction

DNA was extracted using the boiling method as described by Hitchins et al. (2004). Cells were harvested by centrifuging overnight pure culture of *Salmonella* spp. Isolates in 2 ml Eppendorf tubes for 2 min at 10,000rpm. The supernatants were discarded. Pellets were resuspended in 100 µl of distilled water, boiled for 10 min and placed on ice cubes for 5 min after which it was centrifuge at 10000rpm. Supernatants were then transferred to fresh Eppendorf tubes and stored at -20^{o}C until further analysis.

Determination of virulent genes of *Salmonella* spp.

Oligonucleotide primers for *fli*C, *inv*A, and *sef*A virulence genes synthesized by Bimers.net, Germany were employed. PCR was conducted in thermocycler (Mastercycle-Eppendorf, Vapour Product, Germany) in a volume of 25 µl 10xPCR buffer, 25nM $MgCl_2$, 2.5DNTPs each of appropriate 0.1 primer, 0.1µl Taq polymerase, 10 µl of appropriate DNA preparation and 13.4 µL distilled water. Amplification following an initial denaturation at 94^{o}C for 5 minutes was performed in 35 cycles at 94^{o}C for 15s, 55^{o}C for 20s and 72^{o}C for 30s. A final extension was done for 7 min at 72^{o}C. An 8µl aliquote of the PCR product mixed with a loading dye (10mM, EDTA, 10% glycerol, 0.015% bromo phenol dye and 0.017% SDS, made up to 100 ml) were checked using Portable Gel hood built in Blue LED (470nm) by Royal Biotech/Biolympics,1.5% agarose gel at a constant voltage and 1X TBE for approximately 1h. They were visualized by Ethidium bromide staining and photographed under ultraviolet light. The ladder used is 1kb base pair ladder from thermo scientific (Eruteya and Odunfa, 2014).

RESULTS

Nutritional Composition of Raw and Ready-to-eat Snails Available in Port Harcourt

Proximate analysis done on representative raw and ready-to-eat snail samples showed varying percentages in nutritional composition. Raw snail had more moisture (78.33 %) compared to the ready-to-eat (66.67%) while in terms of crude protein, ready-to-eat snail meat had (12.03 %) as opposed to the 10.01% recorded for raw snail meat. Likewise, ready-to-eat snails had a relatively higher carbohydrate content (11.61%) when compared to the raw sample (3.19%) (Table 1).

Table 1: Average nutritional composition of the examined raw and ready-to-eat snails

Parameter	Percentage (%) Composition	
	Raw Sample	Ready-to-eat Sample
Ash	2.67	3.13
Moisture Content	78.33	66.67
Crude Lipid	2.50	1.76
Crude Protein	10.01	12.03
Crude Fibre	3.20	4.80
Carbohydrate	3.29	11.61
Ash	2.67	3.13

Occurrence of *Salmonella* spp. in the various samples studied

The number of *Salmonella* spp. isolated from the various samples and sampling areas are as presented in Table 2. The result showed that all raw samples had *Salmonella* spp. For the ready-to-eat snail samples, *Salmonella* was detected in samples sourced from Rumuokoro and Oyigbo only.

Table 2: Occurrence of *Salmonella* spp. in the various raw and ready-to-eat snail

Source	No of samples collected	Raw Snail No. positive (%)	Ready-to-eat Snail No. positive for E. coli (%)
Choba	10	10 ((100%)	0
Rumuokoro	10	10 (100%)	3(30%)
Oyigbo	5	5 (100%)	2(40%)
Total	25	25 (100%)	5 (20%)

Raw snail samples sourced from Choba market showed different *Salmonella* load ranging from 3.32 to 5.04 $^{log}{}_{10}$CFU/g. However, all the ready-to-eat snail sourced from this area were free of *Salmonella* as growth was not observed on *Salmonella -Shigella* agar plates (Figure 1)

Raw snail samples sourced from Rumuokoro market showed different load of *Salmonella* species which ranged from 4.04 to 6.04 $\log_{10}$CFU/g. However, ready-to-eat snail sourced from this location had just three samples showing *Salmonella* growth recorded on *Salmonella-Shigella* agar plates, with load ranging from 3.53 ^{to} 3.63 $\log_{10}$CFU/g (Figure 2).

Raw snail samples sourced from Oyigbo market had *Salmonella* count ranging from 4.71 to 6.67 $\log_{10}$CFU/g, while ready-to-eat snail had counts of 3.69 and 3.51 $\log_{10}$CFU/g respectively (Figure 3).

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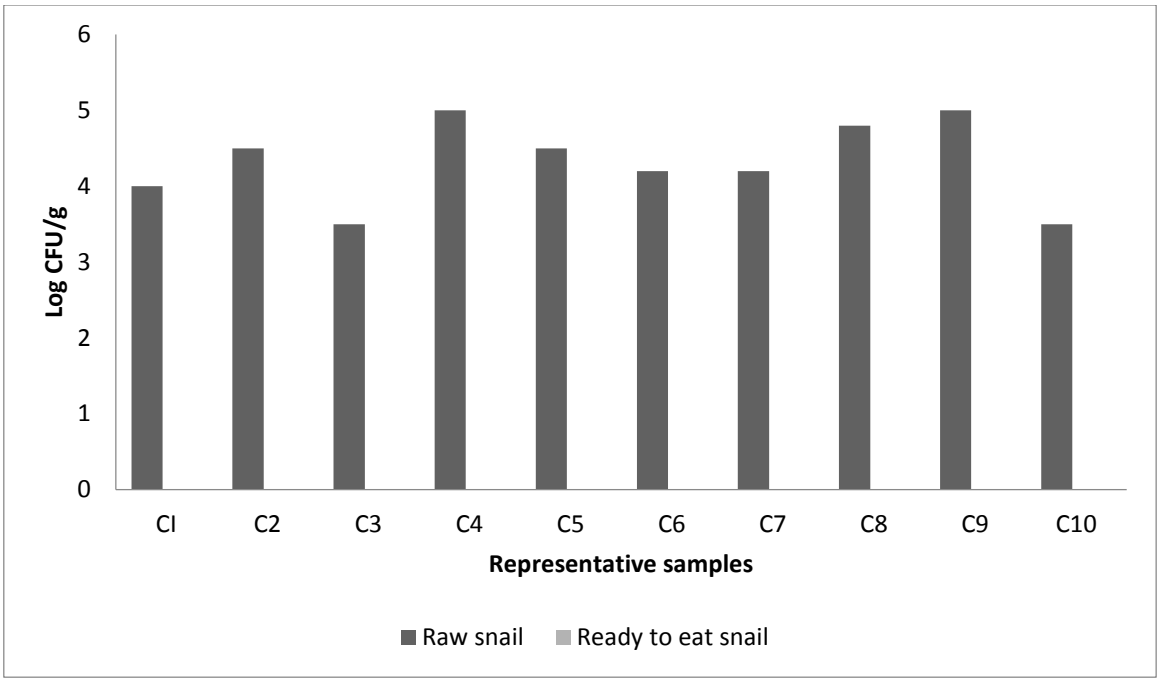

Fig 1: Occurrence of *Salmonella* spp. in raw and ready-to-eat snail sampled from Choba market

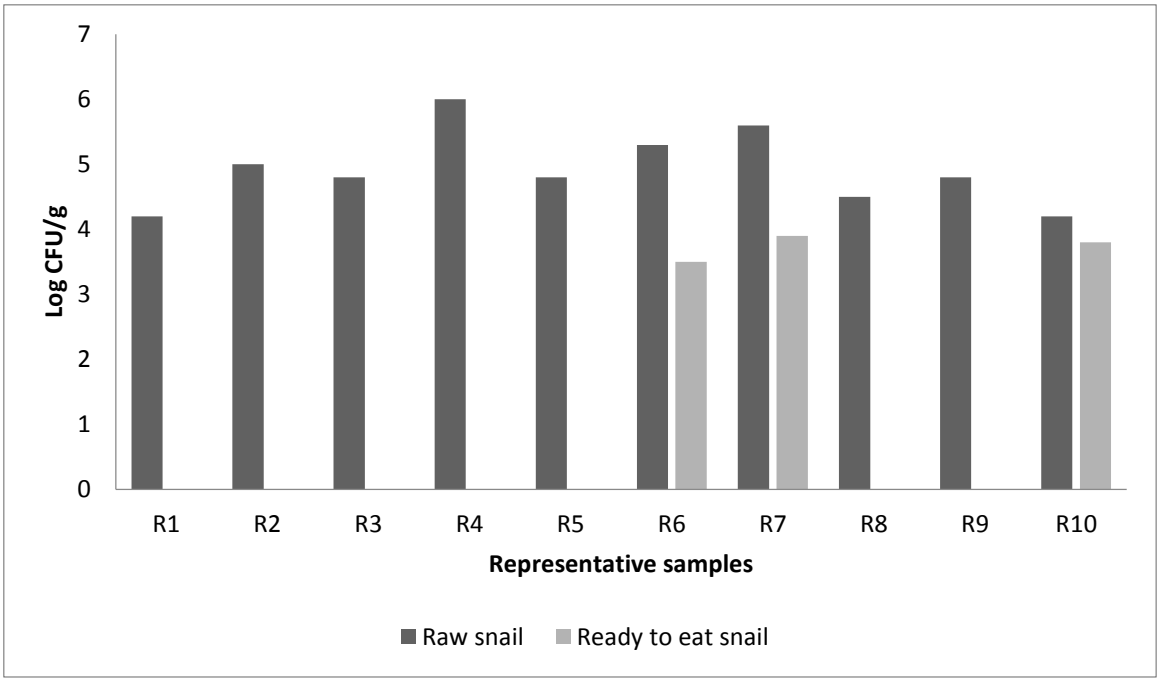

Fig 2: Occurrence of *Salmonella* spp. in raw and ready-to-eat snails sampled from Rumuokoro market

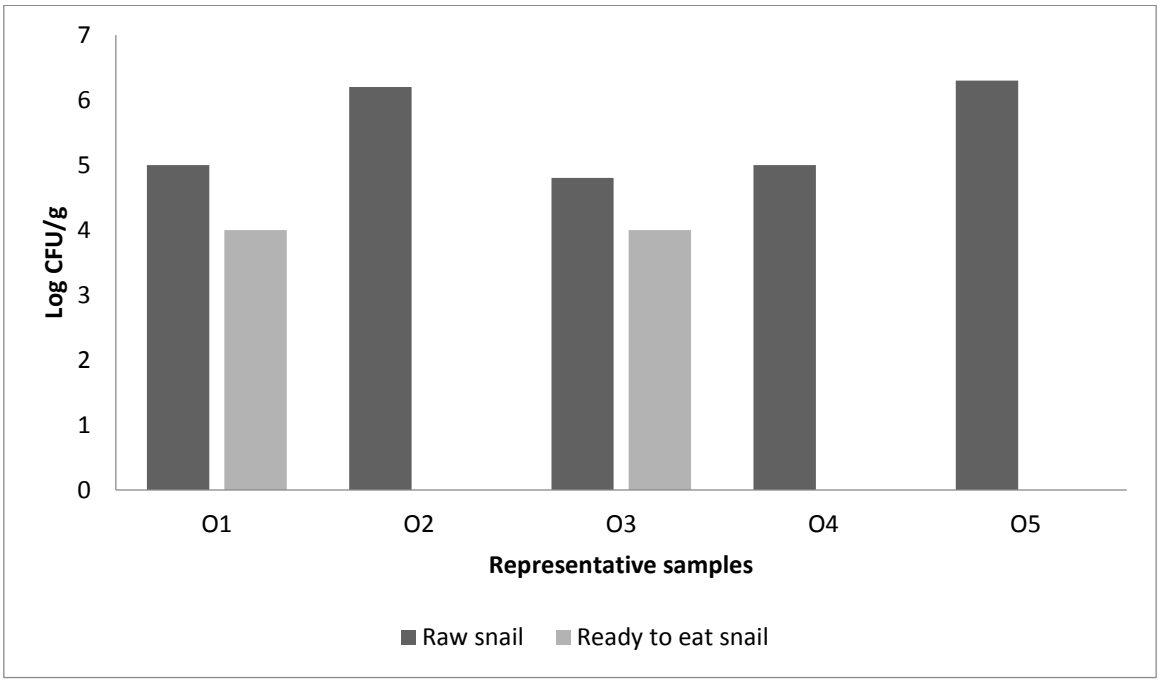

Fig 3: Occurrence of *Salmonella* spp. in raw and ready-to-eat snail sampled from Oyigbo market

Antibiotic susceptibility profile of *Salmonella* isolated from the three markets

The antimicrobial susceptibility pattern of all *Salmonella* isolated from both raw and ready-to-eat snails showed different sensitivities to the different readily available Gram's negative antibiotics. All the isolates showed 100 % resistance to augmentin, ciprofloxacin and cefuroxime and 100% susceptibility to ciprofloxacin and ofloxacin across locations (Table 3).

Table 3: Percentage (%) antibiotics sensitivity of *Salmonella* species isolated from the three markets

Antibiotics	Choba (n=20)			Rumuokoro (n= 23)			Oyigbo (n=12)			Overall sensitivity report across all markets (N=53)		
	R	**I**	**S**	**R**	**I**	**S**	**R**	**I**	**S**	**R**	**I**	**S**
CRX	20(100)	0(0)	0(0)	23(100)	0(0)	(0)	12(0)	(0)	0(0)	55(100)	0(0)	0(0)
AUG	20(100)	0(0)	0(0)	23(0)	0(0)	0(0)	12(0)	0(0)	0(0)	55(100)	0(0)	0(0)
NIT	4(20)	4(20)	12(60)	3(13.1)	1(4.3)	19(82.6)	2(16.7)	1(8.3)	9(75)	9(16.4)	6(10.9)	40(72.7)
CPR	0(0)	0(0)	20(100)	0(0)	0(0)	23(100)	0(0)	0(0)	12(100)	0(0)	0(0)	55(100)
CAZ	20(100)	0(0)	0(0)	23(100)	0(0)	0(0)	12(100)	0(0)	0(0)	55(100)	0(0)	0(0)
GEN	4(20)	12(60)	4(15)	7(30.5)	13(56.5)	3(13.0)	5(41.7)	5(41.7)	2(16.6)	16(29.1)	30(54.5)	9(16.4)
CXM	11(55)	2(10)	7(35)	18(78.3)	1(4.3)	4(17.4)	8(66.7)	1(8.3)	3(25.0)	37(67.3)	4(7.3)	14(25.4)
OFL	0(0)	0(0)	20(100)	0(0)	0(0)	23(100)	0(0)	0(0)	12(100)	0(0)	0(0)	55(100)

Key: AUG; Augmentin, NIT; Nitrofurantion, CPR; Ciprofloxacin, CAZ; Cetazidime,; GEN.; Gentamicin, CXM.; Cefixime, OFL.; Ofloxacin, CTR.; Cftriaxone, ERY; Erythromycin, CXC.; Cloxacillin. CRX; Cefuroxi; (0-13mm- Resistant (R), 14-16mm - Intermediate (I), 17> Sensitive)

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Prevalence of virulent genes among *Salmonella* species isolated from raw and ready-to-eat snails

Five (5) *Salmonella* of the total number isolated representing 9.1% had each of *fliC* gene (Plate 1) and *InvA* gene (Plate 2) with none showing band for *sefA* gene.

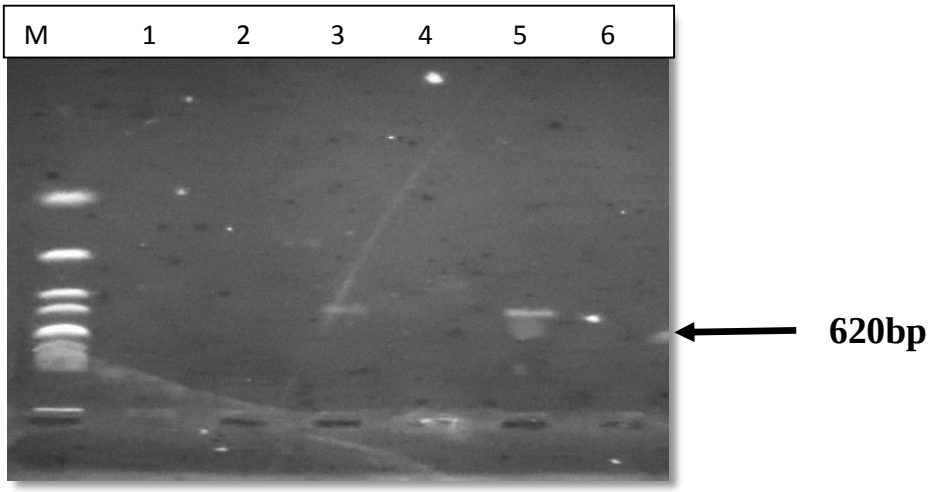

Plate 1: Agarose gel of polymerase chain reaction amplification products of *fliC* virulence gene (620bp) from *Salmonella* isolated from raw snail in Rumokoro and Oyigbo. M is 1kb DNA ladder. Samples 1, 2, 4 and 6 are negative samples while samples 3 and 5 are positive samples

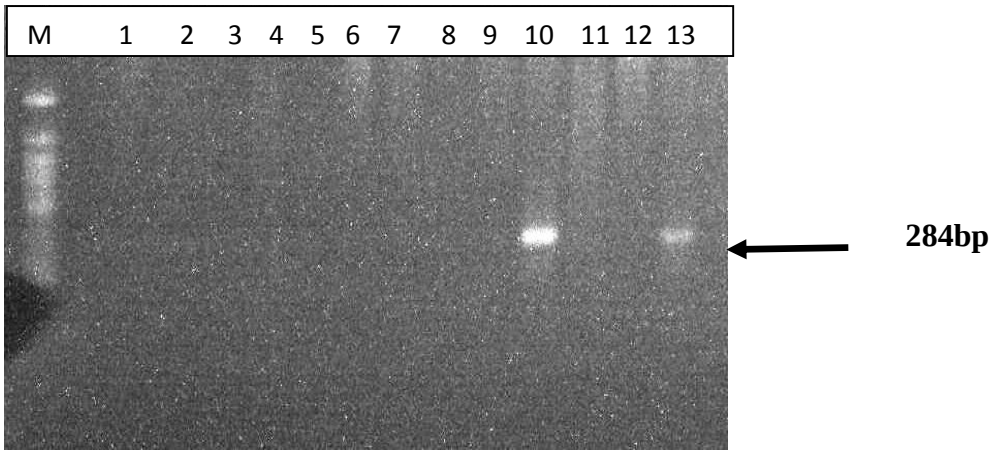

Plate 2: Agarose gel of polymerase chain reaction amplification products of *inv*A virulence gene (284 bp) from *Salmonella* species isolated from raw snail in Rumokoro and Oyigbo. M is 1kb DNA ladder. Samples 1 - 9, 11 and 12 are negative samples while samples 10 and 13 are positive samples

DISCUSSION

Snail meat as a delicacy commonly consumed in south-south Nigeria and vended most times on street, there is a high chance of food poisoning arising from its consumption, owing to improper processing or even cross contamination during transportation to and at point of sales. The inevitable contact snail makes with soil during crawling make them carrier of soil borne pathogens and considering the use of snails as food, may pose risk to consumers. In the present study, the entire raw snail sampled were positive for *Salmonella*. Ready-to-eat samples from two out of the three sampled locations were positive for *Salmonella.*

The *Salmonella* counts of raw snail ranged from 3.32 to 5.04 log_{10}CFU/g, 4.04 to 6.04 log_{10}CFU/g and 4.71 to 6.67 log_{10}CFU/g, from samples purchased from Choba, Rumuokoro and Oyigbo, respectively. Findings in the present study are comparable to the counts, ranging from 2.91±3.19 to 7.39±0.45 log_{10}CFU/g and 0.4 to 3.56 log_{10}CFU/g reported by Nyoagbe et al. (2016) and Daminabo et al. (2020) in raw snails sold in markets and breeding farms in Greater Accra Region and markets across Port Harcourt, respectively. Differences may be attributed to differences in the farms from which the snails were purchased by the traders, since snails feed on debris. The overall prevalence of *Salmonella* species in raw samples from all the markets was 25 (100 %). The overall prevalence in this study is not in agreement with the study by Adagbada et al. (2011) who recorded 40% prevalence in their study.

The ready-to-eat snail samples sourced from Choba had no *Salmonella* while *Salmonella* counts ranged from 3.53 to 3.63 log_{10}CFU/g for Rumuokoro samples, and 3.69 and 3.51 log_{10}CFU/g for the two samples from Oyigbo. The overall prevalence of *Salmonella* species in the ready-to-eat sample was 20% (n=5). The difference in *Salmonella* counts between the raw and ready-to-eat snail samples was not statistically significant. Since raw snails feed on debris, they require proper cooking to be fit for consumption. But it appears that the samples were not properly processed through heating or where not handled under the best sanitary condition by the vendors.

The results of the antibiotic test showed that screened *Salmonella* species were 100% resistant to at least one of the antibiotics tested. The *Salmonella* isolates showed 100 % resistance to augmentin, cefuroxi and cetazidime and 100% susceptible to ciprofloxacin and ofloxacin across locations. However, a 72.7 % (n=40), 16.4 % (n=9) and 25.4% (n=14) *Salmonella* susceptibility was recorded across the markets for nitrofurantoin (NIT), gentamycin (GEN) and cefixime (CXM), respectively. The total sensitivity of *Salmonella* to ciprofloxacin and ofloxacin recorded in the present study is in agreement with the 100% resistance reported by Daminabo et al., (2020) in Port Harcourt. The 100% (n=55) resistance recorded for *Salmonella* against augmentin in this study agrees with result for augmentin 100% (n=3) reported by Adebayo-Tayo et al. (2012) but differed from result reported by Daminabo et al. (2020) for *Salmonella* resistance to augmentin,18.1% (n=19) and also the report of *salmonella* resistance to gentamycin of 100%(n=105) as against 29.1% (n=16) in the present study. Onifade and Aiyenuro (2018) reported that augmentin and ceftriaxone were the least effective against *Salmonella* and other isolates from snails.

Five (5) *Salmonella* species produced the expected 620bp and 284bp against *fliC* and *inv*A genes, respectively representing 9.1 % occurrence of both genes among *Salmonella* isolated from raw and ready-to eat snails. However, none of the *Salmonella* isolated produced bands against *sef*A gene conferring resistance to any of the antibiotics tested. Sallam and El-Wakiel (2012), in their study reported higher prevalence of *fliC* genes (52.94%) in *Salmonella* species isolated from broilers in Egypt. Their result is comparable to the findings of this study which however revealed a lower occurrence of *fliC* genes (9.0%). A number of authors in Nigeria have also confirmed the presence of *inv*A gene in *Salmonella* from milk and milk products, food samples and poultry in Osun, Lagos and Plateau States, respectively (Olufunke et al., 2014; Anejo‑Okopi et al., 2016; Smith et al., 2015). Other authors in Gujarat, Malaysia and Burkina Faso have also detected *inv*A gene in 66 to 100% *Salmonella* isolated from pork and slaughter environment, retail beef and human and street foods (Chaudhary et al., 2015; Thung et al., 2018; Nikiema et al., 2021).

CONCLUSION

This study has shown that raw and ready-to-eat snails sold in Port Harcourt are highly

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contaminated with *Salmonella* spp. Results of antibiogram revealed that all *Salmonella* species were resistant to at least one of the antibiotics tested. Virulence genes *fli*C and *inv*A were detected in the *Salmonella* species with *fli*C gene more prevalent. The presence of *Salmonella* species in ready-to-eat snails obtained in this study strongly suggests the urgent need to improve on the process control for consumers' and public health safety.

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