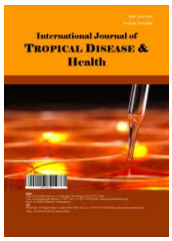

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Isolation of Diarrhea Causing Organisms (*Salmonella and Shigella*) from Selected Seafood

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Authors' contributions

This work was carried out in collaboration among all authors. Author OAW designed the study, wrote the protocol and wrote the first draft of the manuscript. Authors CAW and ASN managed the analyses of the study. Author ASN managed the literature searches and performed the statistical analysis. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The study investigated the presence of entities causing diarrhea (*Shigella & Salmonella*) from some selected seafood.
Study Design: The study adopted a completely randomized experimental Design.
Place and Duration of Study: Department of Medical Microbiology, Rivers State University Teaching Hospital (RSUTH), between January 2020 and February 2020.
Methodology: Simple random technique was employed to collect sufficient quantities of five different fresh raw seafoods (shrimp, periwinkle, crab, sardine fish and mudskipper) across fish harbors and fish markets (Nembe waterside, Abonema Wharf and 1 Fish Market) in Port Harcourt, and were evaluated for bacteriological quality. Sample collection was a cross-sectional type. The isolation and identification of isolates were done according to standard bacteriological analytical methods. The study employed Frequency counts, percentages and one- way ANOVA statistics, and

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the analysis was done using SPSS version 23. Although, one-way ANOVA statistics was used to test the hypothesis of the study at 0.05 level of significance, while Tukey's test was used for ranking means.

Results: The finding showed that 53 percent of the isolates (i.e. 8 out of 15 isolates) were characterized as *Salmonella and Shigella*. Also, the result shows that all the seafood evaluated contain unacceptable levels of *Salmonella and Shigella* contamination, which ranged from 1.79 $x10^7$ CFU/g to 2.96 $x10^7$CFU/g. The level of contamination found in the selected seafood is shown in descending order from the highest to the lowest: Sardine> Periwinkle> Shrimps> Mudskipper> Crab. More so, result from the hypothesis showed that there was a significant mean difference in the *Salmonella and Shigella* count amongst selected seafood ($P < .001$).

Conclusion: The results of this study constitute an indicator of fecal contamination in selected seafood from fish markets in Port Harcourt. Amongst others, it was recommended that Government should enforce laws discouraging the dumping of untreated waste into water bodies.

Keywords: Diarrhea; Salmonella; Shigella and seafood.

1. INTRODUCTION

Seafood can become poison (food poisoning) when it is contaminated by pathogenic microorganisms. This can be as a result of fecal contamination of the water body harboring this seafood. In fact, contamination of seafood by some pathogenic microorganism (Salmonella) has been reported in imported and internal market of the United States [1]. Seafood borne illnesses can cause mild to severe symptoms and even death in untreated immunocompromised individuals (children and aged). The major symptoms include; diarrhea, fever abdominal cramps, vomiting etc. Diarrhea is defined by the World Health Organization as having three or more loose or liquid stools per day or as having more stools than is normal for that person. It is usually a symptom of 'gastroenteritis' (inflammation of the lining of the stomach and intestine) and can be accompanied by severe abdominal pain. Diarrhea is generated by several pathological states – most commonly, infection, intestinal disorders, and food poisoning (presence of pathogenic microorganism or its toxin in food). Although the human large intestine ordinarily harbors a huge microbial population, most are bacterial, protozoan and viral. But the agents of diarrhea are not members of this normal gut (intestinal) flora, they acquired through contaminated food or water.

Fish and shellfish appear to be passive carriers of *Salmonella;* they demonstrate no clinical disease and can excrete *Salmonella* spp. without apparent trouble [2]. The contamination of this organism derives from terrestrial sources and fish may serve as a vector for *Salmonella* spp. and other enteric pathogens ([3,4,5]). An outbreak of *Salmonella* spp. Infections following smoked eel (fish) consumption was described in Germany. The consumed eel came from four different local smoke houses, but could be traced back to fish farms in Italy [6]. This outbreak indicates that eel (fish) may be a vector of *Salmonella* spp. and that the smoking process may not eliminate bacterial contamination from raw fish. Also, *Salmonella enterica* was isolated from the stool of a 14-month old boy who suffered from diarrhea, vomiting, and fever for two days. The same isolate was identified from the water of home fish [7]. Fish was the vector of *Salmonella* spp in this case, this implies that fish and shellfish are vectors of food-borne diseases.

Salmonella species are leading foodborne pathogens; causative agents of the most common enteric infections to human. Each year an estimated 1.4 million cases of salmonellosis occur among humans in the United States [8]. Unlike the developing countries, seafood – borne illnesses are well documented in the developed world. Contamination of the water body from rain and storm water run-off, sewage, untreated waste-water, dumping of refuse in water bodies, use of water bodies as toilets etc can result in contamination of seafoods leading to food-borne illnesses.

Port Harcourt, Rivers State, Nigeria is made up of about 1.4million people living in an area surrounded by rivers and mangrove swamps and consumption of seafood is a major part of their daily diet. Considering the geographical location and climate of the region including the presence of slums and a long season of rainfall (over 9months), it has become paramount to investigate the presence of diarrhea causing organisms in some selected seafoods. This is to help evaluate the sanitary quality of seafoods in the target area.

However, the most pathetic thing is not the recorded incidence of food-borne diseases, but that there is no notification or surveillance system for *Salmonella and Shigella* in Nigeria. Individual studies and laboratory records in many specialized hospitals revealed that typhoid fever and bacillary dysentery are endemic in Nigeria ([9,10] and Onile et al., [11]. This lack of proper surveillance system in the country and lack of good hygienic practices especially in the coastal areas and other factors prompted the researchers to undertake the task of testing the microbiological quality of seafood in Port Harcourt, Southern Nigeria.

1.1 Aim and Objectives

The aim of this research work was to investigate the presence of diarrhea causing organisms (*Shigella & Salmonella*) from some selected seafoods. In specific term, the objectives include:

1. To determine the presence of diarrhea causing organisms (*Salmonella and Shigella*)
2. To ascertain the level of *Salmonella and Shigella* count in seafood
3. To compare the *Salmonella and Shigella* count amongst selected seafood.

2. MATERIALS AND METHODS

The following procedure was adopted step-by-step for determining the *Salmonella and Shigella* Count in Selected Seafood.

2.1 Experimental Design

The study adopted a Completely Randomized Design (C.R. Design). The C.R. design is an experimental design used for investigating the effect of one independent variable (usually with number of categories) on the dependent variable [12]. This design supports the use of one-way ANOVA statistics for data analysis.

2.2 Sample Collection

Simple random technique was employed to collect sufficient quantities of five different fresh raw seafoods (shrimp, periwinkle, crab, sardine fish and mudskipper) across fish harbors and fish markets (Nembe waterside, Abonema Wharf and 1 Fish Market) in Port Harcourt, and were transported to the laboratory for bacteriological analyses. Sample collection was a cross-sectional type.

2.3 Preparation of Media

The media used for isolation of *Salmonella and Shigella* organisms from seafood's includes: Desoxycholate Citrate Agar, Salmonella-Shigella Agar and Selenite-F broth. The media used for isolation of *Salmonella and Shigella* was not sterilized using autoclave because it is a synthetic media. Instead, the two media used for isolation (Deoxycholate citrate agar and Salmonella-Shigella Agar) were sparing heated using bunsen flame. The Inoculating loop was sterilized by labile material were aseptically rinsed with alcohol and distilled water.

2.4 Sample Processing and Culture

The fresh seafood samples were processed as follows: firstly, the shelled seafood samples (crab and periwinkle) were cracked and the meats (internal organs) were aseptically extracted, while the other fish sample (mudskipper, sardine and shrimp) were thoroughly washed in distilled water. Exactly 10 g of each of the samples were homogenized with 90ml of normal saline in a stomacher blender. The homogenized seafood was used to carry out a 10-fold serial dilution, which produced the following concentration of the homogenized seafood's: 10^{-1} (stock culture), 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}. Although, pre-enrichment was followed by inoculating a loopful of homogenates (stock culture) of each sample into Selenite F broth and incubated at 37^{0}C for 24 hrs.

Next, the media used for culturing include: Nutrient Agar, Salmonella-Shigella Agar and Deoxycholate Citrate Agar using spread plate method, where 0.1ml aliquot of the appropriate diluted samples were inoculated on the Nutrient Agar plates and incubated at 37^{o}C for 24 hrs for Total Heterotrophic Bacterial Count (THBC) while, Salmonella-Shigella Agar (SSA) plates and Deoxycholate Citrate Agar (DCA) plates were used for Salmonella-Shigella count. The plates with counts within the microbiological range of 30–300 were recorded while, plates with confluent growths were not counted.

Further, the selectively enriched sample (pre-enriched in Selenite-F broth) were sub-cultured onto DCA and SSA plates, still to be used for Salmonella-Shigella Counts and as substitute means to isolate *Salmonella and Shigella* if they were unable to grow ordinarily without pre-enrichment. Duplicate plating was used for all culturing carried out in this research work.

2.4.1 Isolation and characterization of *Shigella* and *Salmonella* in selected seafood

After incubation of the original sample at 37^0C for 24hrs, growth was observed. For isolating a pure culture, fifteen discrete colonies showing different cultural characteristic from the original incubated plates were picked using a sterile wire loop and sub-cultured on a fresh Nutrient Agar and Deoxycholate Citrate Agar (DCA) plates which was incubated at 37^0C for 24hrs to obtain pure culture. Plates that showed confluent growth were not used to make slants. Pure colonies from the sub-culture plates were stored on Nutrient Agar slants, prepared in a screw-capped McCartney bottle and incubated to inhibit excessive growth and these were used for further experiments.

The suspected isolates were identified through cultural morphology (macroscopic examination), gram reaction (microscopy) and biochemical test such as: motility test, indole, oxidase, citrate, catalase, urease, coagulase, H_2S, Acid, Gas etc. as described by Cheesbrough [13].

2.5 Data Analysis

The study employed Frequency counts, percentages and one- way ANOVA statistics, and the analysis was done using SPSS version 23. Data collected for the study was analyzed using one-way Analysis of Variance (ANOVA) while, Tukey pair wise test was used for ranking means.

3. RESULTS AND DISCUSSION

The results of the study were presented using charts and tables below.

3.1 Determination of Salmonella-Shigella Count in Selected Seafood

Salmonella-Shigella Count (SSC) at 10^{-4} dilution was used for Colony Forming Unit (CFU) calculation, because they were within the microbiological acceptable range of colony count (30-300 colonies). The Salmonella-Shigella Count (SSC) ranged between $2.96x10^7$cfu/g in sardine samples and $1.79x10^7$cfu/g in crab sample. The result shows that the incidence of Salmonella-Shigella Count present in seafood is highest in sardine fish followed by periwinkle and shrimps, crab had the lowest level of bacterial contamination (Table 2).

3.2 Comparison of Salmonella-Shigella Count amongst Selected Seafood

To test the hypothesis, ANOVA statistics was used to test for difference among the selected seafoods. While, Tukey test was used for ranking the mean among selected seafood sample.

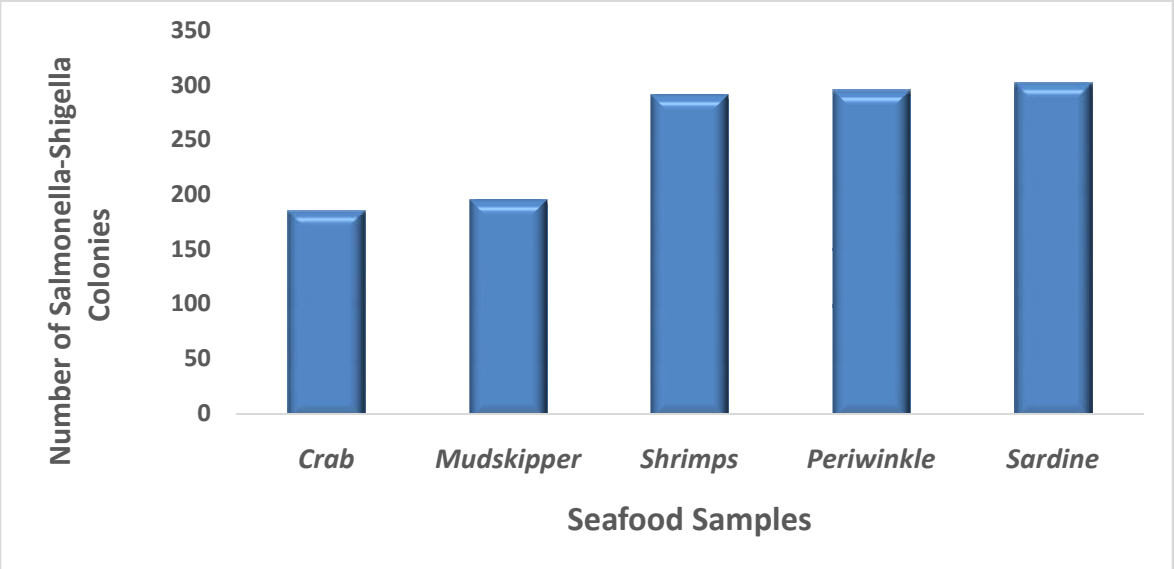

Fig. 1. Incidence of *Salmonella Shigella* organisms in selected seafood

Table 1. ANOVA Test showing the mean difference of *Salmonella Shigella* Count (SSC) among selected seafood sample

	Sum of squares	Df	Mean square	F	Sig. (P value)
Between Groups	2.719	4	.680	74.210	.000
Within Groups	.046	5	.009		
Total	2.765	9			

The results from Table 1 indicated a significant difference in the Salmonella Shigella contamination among selected seafood ($F_{4,9}$ = *74.2; P < .001*). As a result of this, Tukey test was used for ranking the mean among selected seafood sample.

Results from the Tukey pairwise test in Table 2 shows that there is no significant mean difference when comparing the Salmonella-Shigella count between seafood organisms with same letter (*P > .05*), however, a significant mean difference exists in comparing the Salmonella Shigella count between organisms with different letters (*P< .001*).

3.3 Discussion

The finding showed that 53 percent of the isolates (i.e. 8 out of 15 isolates) were characterized as *Salmonella and Shigella*. Others include; *Pseudomonas, Escherichia, Vibrio, Proteus, Enterobacter* and *Klebsiella*. The result revealed that the organisms are mainly gram negative bacteria from the Enterobacteriaceae family. This trend of bacterial contaminants is in consonance with the following studies; Wandili, et al. [14,15]. Also, the high percentage of *Salmonella and Shigella* isolated from seafood in this study was corroborated by the following studies; Kumar et al. [8], Adedeji and Ibrahim [16] and Bukola et al., [17].

Also, the present study demonstrates a considerable increase in the prevalence rate of pathogenic and opportunistic microorganism in seafood in Port Harcourt, Rivers State. The result shows that all the seafood evaluated contain unacceptable levels of *Salmonella and Shigella* contamination, which ranged from 1.79 x10^7 CFU/g to 2.96x10^7CFU/g. The contamination level exceeds the acceptable limits for shellfish. The International Commission on Microbiological Specification for Food (ICMSF) and US Food and Drug Administration (FDA) have suggested a maximum microbial count IPC of not greater than 1×10^2cfu/g and *Salmonella-Shigella* Count of zero cfu/g for approved harvest area or water. While, for unknown topical water such as the ones within the researchers' location (Nembe & Timber Rivers) the standard is much lower [18], [19]. The above report agrees with the report of Adedeji & Ibrahim [16] and Bukola et al., [17] who also observed unacceptable levels of contamination in seafood's in different part of the country Nigeria. This high level contamination of seafood's depends on the amount of pollution in the growing water. The big concern in this part of the country is that, there are no state shellfish/seafood control authorities to monitor the level of bacterial and other pathogenic organism in water and seafood. Also, water bodies or growing areas in this part of the country are not classified (as approved, conditionally approved, restricted or prohibited for seafood harvesting). This makes it difficult to trace any illness back to its sources.

Table 2. Tukey Test for ranking of *Salmonella Shigella* Count (SSC) (Mean ± SE) of Selected seafood sample at 10^7 CFU/g

Seafood sample	(Mean ± SE)
Crab	$1.79 \pm .07^{a}$
Mudskipper	$1.89 \pm .03^{a}$
Shrimps	$2.85 \pm .06^{b}$
Periwinkle	$2.89 \pm .05^{b}$
Sardine	$2.96 \pm .01^{b}$

Each value is the mean of 2 replicates. Means of seafood sample in each column followed by the same letter are not significantly different by Tukey's test while, seafood sample having mean with different letters are Significant

Port Harcourt also called the "Garden City" is one of the areas where the natural environment has continued to deteriorate. Port Harcourt is a very populous city surrounding by rivers and mangrove swamps with seafood which is a major part of its daily cuisine. Unfortunately, the water bodies holding these seafood has suffered from lax sanitation including improper waste management, excretal disposal into rivers, lack of hygiene education (people bathe, wash and defecate in rivers), poor drainage channels and lack of waste water treatment. Excessive rainfall is also common in this region and intermittent

flooding usually occur too. All these activities result in contamination of the water bodies and corresponding contamination of seafood.

Consequently, the ingestion of these asymptomatic poisoned seafood by members of the public results in food poisoning with diarrhea a major symptom of this food bore illness. This is corroborated by Novotny et al., [2]; Metz, (1980); and Chattopadhyay [5] that seafood is a vector of enteric pathogens. Above all, the organisms isolated have health implications for man, some include: severe infantile diarrhea, typhoid fever, shigellosis, cholera, septicemia, and neonatal meningitis, wounds and burn infection, nosocomial infection and other opportunistic illness ([20,21,22]).

According to Kumar et al. [8] studied the "Distribution of *Salmonella* in seafood in India. A total of 417 seafood samples were collected over 2003-2006 from fishing harbors and fish markets of Cochin (India). Samples included whole body parts of fish, shrimp, lobster, squid, octopus, cuttlefish and soft muscle parts of crab, clam, oyster and mussel. The result that *Salmonella* was present in seafoods with clam (34.2%), mussel (31%), fish (28.2%) and shrimp (26.7%), while lobster (4.7%) and crab (9.6%).

According to Bukola et al. [17] studied the "Bacteriological and Proximate Analysis of periwinkles from two different creeks in Akwa Ibom State, Southern Nigeria. The result shows that all the periwinkles contain unacceptable levels of bacteria ($1.2x10^8$cfu/g) and coliforms count ($1.1x10^6$cfu/g). The organisms present include: *E. coli, Proteus* spp. *Salmonella, Pseudomonas, Bacillus, Micrococcus* and *Enterobacter. Salmonella* and *Pseudomonas* have the highest rate with *Proteus* been the least encountered.

Esomunu et al., (2012) studied "Enteric pathogens and diarrhea disease potentials of water sources in Ahiazu Mbaise, Eastern Nigeria", Imo State. Water samples were collected from boreholes, underground tanks and streams and subjected to standard microbiological analysis. The result of total heterotrophic bacterial count and coliforms ranged between $2.0x10^5$ – $4.8x10^3$ respectively. The isolates occurred includes *E. coli* (50%), *Salmonella* spp. (100%), *Shigella* spp. (100%), *Vibrio* spp. (20%), *Proteus* spp. (30%), *Klebsiella* spp. (80%), *Enterobacter* spp. (50%) and *Streptococcus* spp. (50%).

According to Adedeji and Ibrahim [16] reported unacceptable bacterial contamination of fresh shrimps offered for sales at fish Markets in Ibadan, South Western Nigeria. The total heterotrophic count ranged from $7.6x10^7$cfu/ml to $1.38x10^8$cful/ml, these were high exceeding the limit of $1.0x10^2$cfu/ml accepted microbial count and zero cfu/ml limit of coliform count. The following organisms were present: *Enterobacter, Salmonella, Shigella, Micrococcus, Flavobacterium, Staphylococcus,* and *Bacillus.* They reported that under no condition, should the shrimp product be consumed without any form of pre-treatment because they might serve as source of infection for consumers.

Furthermore, [14] studied "characterization of salmonella isolated from Nile Tilapia along Lake Victoria Beaches in Eastern Kenya. Sample of 120 fish specimen were collected and 63 were positive for various bacterial isolates. *Shigella* spp. (39.6%) was the most isolated Enterobacteriaceae followed by *Salmonella* (31.7%), *E. coli* (25.3%), *Proteus* (1.58%) and *Enterobacter* (1.58%).

4. SUMMARY

The following are the major findings of the study:

1. *Salmonella and Shigella* was the most prevalent microorganism (53%) found in the investigated seafood.
2. The Salmonella-Shigella count ranged from 1.79 $x10^7$ CFU/g to 2.96 $x10^7$CFU/g. The level of contamination found in the selected seafood in descending order: Sardine> Periwinkle> Shrimps> Mudskipper> Crab.
3. The result showed that a significant mean difference in the Salmonella Shigella contamination among selected seafood.

5. CONCLUSION

The study revealed that the level of *Salmonella and Shigella* contamination in selected seafood is beyond the acceptable microbiological standard and thus calls for intervention by responsible authorities. More so the presence these pathogenic bacteria (*Salmonella and Shigella*) in seafood reveals the poor sanitary conditions of water body where the seafood samples were harvested.

6. RECOMMENDATIONS

From the results of the study and considering the public health implications, attention should be given to the following:

- The Government through the Ministry of Health should constitute a reliable surveillance system (like State Seafood Control Authority) to monitor the contamination of seafood and water bodies. As well as to regulate and prevent disease outbreaks especially during floods and other natural disasters.
- Sanitation should be taken seriously especially in riverine communities where seafood are harvested for sale.
- Government should provide basic amenities to some of the fishing habours and such as clean drinking water, proper sanitary system.
- Government should enforce laws discouraging the dumping of untreated waste into water bodies.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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