

Multi-Drug Resistant and Plasmid-Mediated Quinolone-Resistant *Salmonella enterica* isolated from Shellfish in Iko Creek, Nigeria.

Udoekong, Nsikan. S.¹ Akan, Donald. O.² James, Iniobong.I.,¹ Agbo, Bassey. E.³, Umoh, Anthony.⁴

¹ Science Technology Department, Akwa-Ibom State Polytechnic, Ikot Osurua, AKS, Nigeria;

² College of Food Science and Technology, CSUFT, Hunan, China

³ Department of Microbiology, University of Calabar, Calabar, Nigeria.

⁴ Department of Medical Microbiology/ Parasitology, College of Health Sciences, University of Uyo, Uyo, Nigeria.

Corresponding author's e-mail: nsybeat@gmail.com

Abstract

Aim: This study investigated the occurrence of multi-drug resistant (MDR) and Plasmid-Mediated Quinolone Resistant (PMQR) genes in *Salmonella enterica* isolated from shellfish in Iko Creek, Nigeria. **Study design:** This is an experimental research that studied *Salmonella enterica* isolated from shellfish to determine its association with MDR and PMQR which results in drug resistance and treatment failure using standard microbiological and molecular methods. **Place and duration of study:** The study was carried within Iko Creek, South-South Nigeria for a period of one year (March-July and August to February 2017). **Methodology:** A total of 160 shellfish samples (80 clams and 80 oysters) were analyzed for the presence of *Salmonella enterica* using standard bacteriological methods. In all, 22(52.4%) were positive for clams while 20(47.6%) for oysters. The bacterium was identified using phenotypic methods. The antibiotics used for the antibiotic susceptibility profiles were amoxicillin, quinolones, aminoglycosides, macrolide and sulfonamide. Confirmation of the isolates was done by DNA sequencing of 16S rRNA gene; results obtained were then compared with that in the gene bank. The isolate was identified as *Salmonella enterica*. **Results:** The mean heterotrophic bacterial counts (THBC) in clams ranged from 5.25×10^6 to 3.71×10^7 (Cfu/g) in the dry season and 3.05×10^6 to 2.33×10^6 (Cfu/g) in the rainy season. The mean THB counts in oyster was 3.27×10^8 to 1.59×10^8 (Cfu/g) in the dry season and 2.54×10^7 to 4.39×10^6 (Cfu/g) in the rainy season. *Salmonella enterica* showed resistance to all the antibiotics tested except gentamicin. Seventy-one (71) percent of the isolates in this study expressed the *qnrB* genes. **Conclusion:** The presence of *qnrB* genes and multi-drug resistance (MDR) in the isolate's gene according to this study is an indicator of treatment failure with antibiotics.

Key words:-*Salmonella enterica*, shellfish, Iko Creek, multi-drug resistance, plasmid-mediated quinolone-resistance.

1.0 Introduction

Salmonellae are intestinal bacteria and are frequently found in effluents, human waste matter as well as any material exposed to faecal contamination [24]. *Salmonella enterica* have been adjudged

as the primary agents of food-borne illnesses globally because of its involvement in food-borne illnesses and intoxication [20]. Shellfish refers to exoskeleton-bearing aquatic invertebrates that are consumed globally. They include the molluscs (oysters, clams, scallops, mussel and periwinkles), crustaceans (lobsters, crabs, shrimps, crayfish) and echinoderms (sea cucumber, sea urchins, seastars and sand dollars)[27]. Shellfish are very nutritious offering a good source of omega-3 fatty acid, vitamin B12, minerals and micronutrients. It is recognized as a healthy dietary component and a cheap source of income for the coastal populace [25].

Shellfish being filter feeders accumulate pathogenic bacteria, viruses and toxic chemicals from the surrounding water into their body [22]. In addition, *Salmonella* may contaminate shellfish during harvesting, storage and processing [3]. Human salmonellosis arising due to the traditional consumption of raw or partially cooked shellfish are well known to constitute health problems globally [7;13]. Arising from this, there is an estimated 80.3 million cases of food-borne salmonellosis globally (with over 155,000 deaths) [17]. Antimicrobial drugs have been used for the treatment of infections caused by microorganisms until the emergence of antibiotic resistant bacteria in foods from animal origin. A study by Newell *et al.* [21] reported a horizontal transfer of resistance genes between bacteria from shellfish, humans and animals through the food chain. Several treatment options for salmonellosis in humans have failed due to the emergence of both chromosomal and plasmid-mediated resistance against the commonly used antimicrobials [11]. This study is aimed at identifying multi-drug resistance (MDR) and plasmid-mediated quinolone resistance (PMQR) *Salmonella* spp.; and to carryout antibiotic susceptibility profiles on the isolate to aid clinical and health workers in their selection of drugs for treatment of foodborne diseases which may arise from consumption of contaminated shellfish.

2.0 Methods:

2.1 Study Area/design

The study is an experimental analysis carried out within Iko Creek, Akwa Ibom State, in the South-South region of Nigeria. It is located between Latitude $4^{\circ}32'$ and $5^{\circ}33'$ North and Longitudes $7^{\circ}25'$ and $8^{\circ}25'$ East [28].

2.2 Sample collection/processing:

A total of 160 fresh unprocessed clams (80) and oysters (80) were harvested from Iko Creek, Eastern Obolo, Akwa Ibom State, Nigeria during the rainy (March – July) and dry seasons (August – February) of the year 2017. The samples were placed in sterile iced-packed coolers and transported to the laboratory for bacteriological examination and analysis within 5 hours. The shellfish were washed individually by scrubbing with sponge in sterile water, and then rinsed in 70% ethanol to remove external dirt and debris. The shellfish were then shucked aseptically with a sterile shucking knife to remove the soft flesh. The flesh were individually dissected into body parts (intestine and gills) using sterile knives and scissors and homogenised using sterile plastic mortar and pestle. A 5g of the shellfish parts was homogenised in 45ml sterile physiological saline for 5 minutes. A ten-fold serial dilution of the samples was carried out and spread plated in duplicate on nutrient agar (Difco Lab.) plates [for total heterotrophic bacterial counts (THBC)] then incubated at 37°C for 24 hours for bacterial growth. The colonies that developed after 24 hours incubation were enumerated and sub cultured on nutrient agar plates to obtain pure cultures.

Pre-enrichment in non-selective liquid medium for isolation of *Salmonella* spp.

A 25g of each shellfish homogenate was transferred into 500ml Erlenmeyer flask containing 225ml of lactose broth (Oxoid, UK) for pre-enrichment at 37°C in an incubator (Kemi, India) for 24 hours.

Enrichment in selective liquid media

From the pre-enrichment, 0.1ml and 1ml was transferred using a sterile pipette into 10ml of Mueller-Kauffmann tetrathionate broth (MKTB) and 10ml of Modified Semi-solid Rapaport-Vassilidis Agar (MSRV) respectively. The inoculated tubes were incubated at 37°C and 41°C for 24 hours [9].

Selective Plating

From the enrichment, a loop full from the incubated broth was transferred onto Xylose lysine Deoxycholate Agar (XLD), Brilliant Green agar (BGA) and incubated at 37°C for 24 hours.

Purification /maintenance of pure culture

Typical bacterial isolates were purified by streak plating on nutrient agar, and incubated at 37°C for 24 hours. The colonies formed were maintained on nutrient agar slants after sub-culturing as pure cultures before preserving in the refrigerator at 4°C for further analysis.

Characterization and Identification of *Salmonella enterica*

The purified colonies were presumptively characterized using the method of [6]. This characterization was based on the cultural, morphological, Gram reaction, motility tests and standard biochemical tests such as; oxidase, urease, indole, Voges-Proskauer, Hydrogen sulphide production, catalase, citrate utilization and sugar fermentation tests. *Salmonella enterica* was confirmed by sequencing the 16S ribosomal RNA of the isolates and matching a 100% similarity with that in the gene bank.

2.3 Antibiotic susceptibility

The susceptibility of the bacterial isolates to antimicrobial agents was carried out using the Kirby-Bauer disk diffusion method [6; 10 and 31]. Commercially procured discs were used for this study. They were; nalidixic acid (30µg), ciprofloxacin (5µg), amoxicillin (25µg), norfloxacin (10µg), trimethoprim-sulfamethoxazole (25µg) and chloramphenicol (30µg), gentamicin (10µg), erythromycin (5µg), streptomycin (10µg) and tetracycline (30µg.). A bacterial lawn was prepared by transferring culture from stock into tubes containing 2.5ml sterile normal saline using sterile inoculating loop and incubated at 37°C for 4 hours. The density of the suspension was compared with barium chloride(0.5 McFarland Turbidity Standard; 1.0×10^8 cfu/µL). A one hundred microlitres (100µl) of the inoculum was flooded onto already prepared and dried nutrient agar plates using spread plate method. The excess inoculum was siphoned using sterile Pasteur pipettes and the antibiotic discs were aseptically placed on the surface of the seeded medium at equal distance using sterile forceps. The seeded plates were incubated inverted at 37°C for 18 hours. The diameter of the zones of inhibition were read and interpreted in accordance with the United States Clinical and Laboratory Standard Institute [6; 16].

2.4 16SrRNA Amplification

The amplification of the 16S rRNA region of the rRNA genes of the isolates was carried out using the 27F: 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492 R: 5'- CGGTTACCTTGTTACGACTT - 3' primers on a AB1 9700 Applied Biosystems thermal cycler with a final volume of 50µl for 35 cycles. The PCR mix used for the amplification was: X2 Dream Taq Master Mix supplied by Inqaba, South Africa (Taq polymerase, DNTPs, MgCl), a 0.4M concentration of the primers and

the extracted DNA being the template. The conditions required for the PCR amplification included: Initial denaturation at 95°C for 5 minutes; denaturation at 95°C for 30 seconds; annealing at 52°C for 30 seconds, extension at 72°C for 30 seconds for 35 cycles and a final extension at 72°C for 5 minutes. The product of this amplification was transferred onto a 1.0% agarose gel at 120V for 20 minutes and a UV trans-illuminator was used for visualization of the bands.

2.5 DNA extraction/Sequencing

The bacterial genome was extracted using boiling method with heating block at 95°C for 20 minutes. Plasmid DNA was extracted using Plasmid mini- prep kit (Inqaba Biotechnology, Pretoria, South Africa). The sequencing of the amplicons was carried out according to the manufacturer's instructions on a 3510 AB1 sequencer using the Big Dye Terminator Kit (Inqaba Biotechnology, Pretoria, South Africa).

2.6 Amplification of *qnr* genes

The *qnrA*, *qnr B* and *qnr S* genes each were amplified on a AB1 9700 Applied Biosystems thermal cycler at a final volume of 50µl for 35 cycles. The PCR Mix was the X2 Dream TaqMaster mix supplied by Inqaba, South Africa (Taq Polymerase, DNTPS, Mg Cl), 0.4M concentration of Primers was used, and lastly, the extracted DNA was used as template. The cycling conditions for amplification were as follows: Initial denaturation for 5 minutes at 95°C; denaturation for 30 seconds at 95°C; annealing at 52°C for 30 seconds; extension at 72°C for 30 seconds for 35 cycles and final extension at 72°C for 5 minutes. The resulting PCR products were analysed by electrophoresis with 1.0% agarose gels in Tris-borate-EDTA buffer (TBE; Gibco, NT, USA) at 120 V for 20 minutes. The gels were stained with ethidium bromide and bands produced were photographed on an ultraviolet light trans illuminator. A molecular weight standard (100 bp and 1000 bp ladder, Promega, Madison, USA) was included on each gel. The primer sequence used for the amplification of *qnr A*, *qnr B* and *qnr C* genes are: *qnr A*/F: 5¹- TTCACGAAGATTTCTCA-3¹

qnr A/R: 5¹-GGCAGCACTATTACTCCCA-3¹

qnr B/ F: 5¹-CCTGAGCGGCACTGAATTTAT-3¹

qnr B/R: 5¹-GTTTGCTGCTTGCCAGTCGA-3¹

qnr S/F: 5¹-CAATCATACATATCGGCACC-3¹

qnr S/R: 5¹-TCAGGATAAACAACAATACCC-3¹

2.7 Data analysis

The Statistical Package for Social Sciences (SPSS 22.0 Inc., Chicago, USA) was used to analyze the data generated from this study. P-values of less than 0.05 ($P < 0.05$) were considered statistically significant.

3.0 Results and Discussions

The mean heterotrophic bacterial counts in clams and oysters from Iko Creek are presented in Table 1. The mean heterotrophic counts of bacteria in Clams ranged from $5.25 \times 10^7 - 3.71 \times 10^7$ (Cfu/g) during the dry season and $3.05 \times 10^6 - 2.33 \times 10^5$ (Cfu/g) in rainy season. The mean count for oysters was $3.27 \times 10^8 - 1.59 \times 10^8$ (Cfu/g) for dry season and $2.54 \times 10^7 - 4.39 \times 10^6$ (Cfu/g) in rainy season. The shellfish samples in this study harbored higher number of *Salmonella enterica* during the dry season than the rainy season. There was no significant difference in the prevalence of the bacterium across the seasons at $p > 0.05$ for both dry and rainy season. Brands *et al.* [5] also isolated *Salmonella* spp. in oysters in the USA and Heinitz *et al.* [12] isolated *Salmonella* spp in seafoods. In a similar study on shellfish by Martinez-Urtaza *et al.* [18], the researchers observed that higher incidence of food-borne diseases from shellfish were as a result of direct contamination of harvest waters with human sewage.

Oysters and Clams are filter-feeders; therefore they accumulate pathogens and toxic chemicals from the surrounding water that they live in. This explains the higher bacterial count in the gills than the intestine from this study. A study by Solomon *et al.* [27] further explained that contamination of the shellfish could also arise during its processing, transportation and storage or from polluted waters [23]. The comparison of the shellfish bacterial counts in the shellfish parts by seasons showed that the mean rank score of the bacterial colonization in dry season were 72.55 for intestine and 64.46 for gills, 42.45 was recorded for intestine while 31.14 was recorded for gills during rainy season. The reason for the high bacterial counts during the dry season is due to the temperature of the harvesting water, pH and salinity. The numbers seemed to reduce in colder waters [5].

194 The food codex of [14] stipulates absence of *Salmonella* and *Vibrio cholerae* in seafood products.
 195 In this study, *Salmonella enterica* was isolated from the shellfish samples and as such, the shellfish
 196 samples are not fit for consumption, unless cooked properly [29]. The antibiotic susceptibility
 197 profiles of the bacterium are presented in Table 2.

198 The bacterium showed the highest resistance of (100%) against nalidixic acid and norfloxacin,
 199 followed by amoxicillin, ciprofloxacin and streptomycin (95.24%), trimethoprim-sulfamethoxazole
 200 (90.48%), chloramphenicol and tetracycline (83.33%), and erythromycin (71.43%). The isolate
 201 showed multiple drug resistance. Results from this study corroborate results from the works of [1]
 202 who observed multiple drug resistance (MDR) organisms in seafoods and wastewaters [2]. A
 203 similar study by Walsh and Toleman [30] showed that the bacterium may interact and exchange
 204 resistance genes in the aquatic environment that might have been transferred from humans to
 205 animals through food and vice versa [21]. Another study by Foley and Lynne [11] described the
 206 isolation of *Salmonella* strain resistant to ampicillin, chloramphenicol, streptomycin, tetracycline
 207 and sulfonamides. Khan *et al.* [15] observed the resistance of *Salmonella* spp. to trimethoprim-
 208 sulfamethoxazole, ampicillin, tetracycline and chloramphenicol. Results from the work of
 209 Bouchrif *et al.* [4] and Minami *et al.* [19] agree with results of this study on the resistance of
 210 isolated *Salmonellas* pp. to streptomycin, nalidixic acid, ampicillin and tetracycline.

211 The distribution of the *qnr* genes among the *Salmonella* isolate is shown on plate 2. Seventy-one
 212 percent of the isolates in this study expressed the *qnrB* genes but not *qnrA* and *qnrS* genes. The
 213 possession of the *qnr* gene by *Salmonella* spp. confers resistance to the quinolones class of
 214 antibiotics as observed in this study.

215 The *qnr* genes being carried on the genetic element such as plasmid that can step up the synthesis
 216 of the *qnr* proteins may bind to the DNA topoisomerase thereby prevent binding by quinolones [8].
 217 The consequences for the possession of the *qnr* genes include increased severity of the infection,
 218 prolonged hospitalization, treatment failure and even mortality [21].

Table 1: The Mean Heterotrophic Bacterial Count (THBC) in shellfish samples (cfu/g)

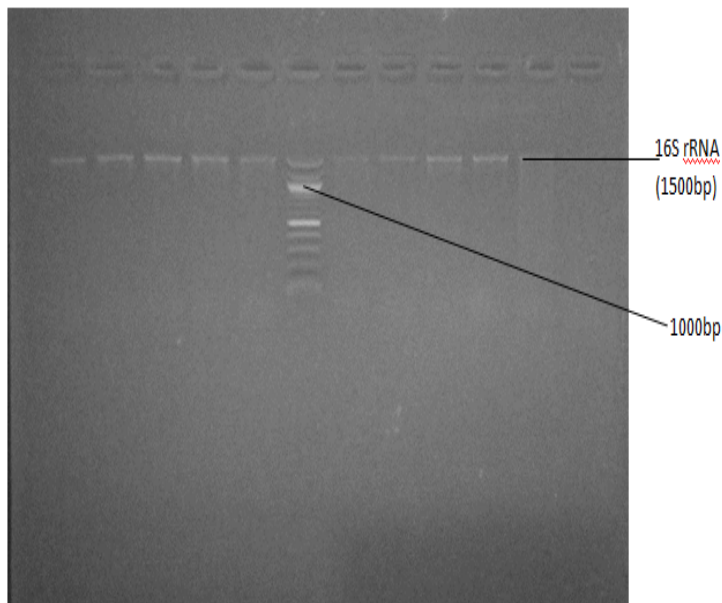
| S/N | Sample | Location | Dry season | | Rainy season | |
|-----|--------|-----------|--------------------|--------------------|--------------------|--------------------|
| | | | Intestine | Gills | Intestine | Gills |
| 1 | Oyster | Iko creek | 3.27×10^8 | 1.59×10^8 | 2.54×10^7 | 4.39×10^6 |
| 2 | Clams | Iko creek | 5.25×10^6 | 3.71×10^7 | 3.05×10^6 | 2.33×10^6 |

Key:-cfu/g – Colony forming unit/gram

Table 2: Antibiotics susceptibility profiles of bacterial isolate

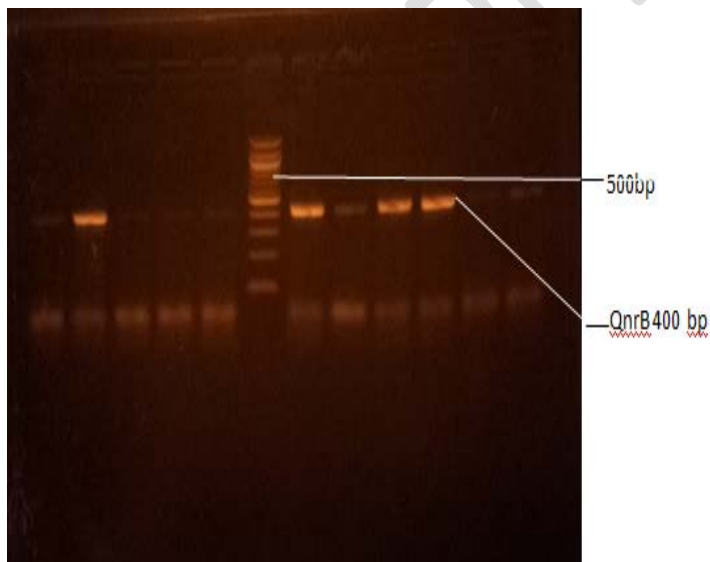
| Antibiotics | Disc content (µg) | Resistant isolates n =42 <i>Salmonella</i> spp. R(%) |
|-------------------------------|-------------------|---|
| Amoxicillin | 25 | 40 (95.24) |
| Nalidixic acid | 30 | 42 (100) |
| Ciprofloxacin | 05 | 40 (95.24) |
| Chloramphenicol | 30 | 35 (83.33) |
| Gentamicin | 10 | 20 (47.62) |
| Erythromycin | 05 | 30 (71.43) |
| Streptomycin | 10 | 40 (95.24) |
| Norfloxacin | 10 | 42 (100) |
| Trimethoprim-sulfamethoxazole | 25 | 38 (90.48) |
| Tetracycline | 30 | 35 (83.33) |

231 1 2 3 4 5 L 6 7 8 9 10 11



233 **Plate 1:** Agarose gel electrophoresis showing the amplified 16S rRNA gene of the bacterial
 234 isolate. Lane L represents the 1,000bp molecular ladder while lanes 2- 10 show the amplified 16 S
 235 rRNA genes positive isolate.

236 1 2 3 4 5 L 6 7 8 9 10 11



238 **Plate 2:** Agarose gel electrophoresis showing the amplified *QnrB* gene (400bp) of the bacterial
 239 isolate. Lane L represents the 500bp molecular ladder, lanes 2, 6, 8 and 9 showed *qnrB* bands
 240 (400bp).

241

Conclusion:

Shellfish samples in this study were contaminated with *Salmonella* isolates possibly from the polluted underlying water in which they were harvested or during storage and processing. The isolates also showed multi-drug resistance against 9 out of the 10 antibiotics tested against them.

The *Salmonella enterica* in this study expressed the *qnr* genes which confers resistance to the quinolone class of antibiotics. The only effective drug for the treatment of *Salmonella* infection in this study was gentamicin. An integrated approach to ensure safer harvesting water for the shellfish should be adopted to avoid food-borne infections from shellfish.

References

1. Adebayo-Tayo, B. C., Odu, N. N., Michael, M. U. and Okonko, I. O. (2012). Multi-Drug Resistant (MDR) organisms isolated from seafoods in Uyo, South-South Nigeria. *Nature and Science*, 10(3): 61-70.
2. Akubuenyi, F. C., Arikpo, G. E., Ogugbue, C. J., Mongeh, J. F. and Akpanumun, E. V. (2011). Antibiotic resistance profile of waste water isolates obtained from the University of Calabar Teaching Hospital and the General Hospital Calabar, Nigeria. *Nigerian Journal of Microbiology*, 25, 2243-2250.
3. Amagliani, G., Brandi, G. and Schiavano, G. (2012). Incidence and role of *Salmonella* in seafood safety. *Food Research International*, 45(2):780-788.
4. Bouchrif, B., Paglietti, B., Murgia, M., Piana, A., Cohen, N. and Ennaji, M. M. (2009). Prevalence and Antibiotics-resistance of *Salmonella* isolated from Food in Morocco. *Journal of Infections in Developing Countries*, 3, 35-40.
5. Brands, D. A., Inman, A. E., Gerba, C. P., Mare, C. J., Billington, S. J., Saif, L. A., Levine, J. F. and Jones, L. A. (2005). Prevalence of *Salmonella* spp. in oysters in the United States. *Journal of Applied and Environmental Microbiology*, 71(2): 893-897.
6. Cheesbrough, M. (2002). *District Laboratory Practices in Tropical Countries*, Part II, United Kingdom: Cambridge University Press, 182 – 187.
7. Cheung, P., Chan, C., Wong, W., Cheung, T. and Kam, K. (2004). Evaluation of two real-time polymerase chain reaction pathogen detection kits for *Salmonella* spp. in food. *Letters in Applied Microbiology*, 39, 509-515.
8. Cheung, T. K., Chu, Y. W. and Chu, M. Y. (2005). Plasmid-mediated resistance to ciprofloxacin and cefotaxime in clinical isolates of *Salmonella enterica* serotype Enteritidis in Hongkong. *Journal of Antimicrobial Chemotherapy*, 56, 586-589.

9. Farmer, J.J., Janda, M., Brenner, F. W. Cameron, D. N. and Brikhead K.M. (2005). Genus I. *Vibrio pacini* 1854, 411AL. In Brenner, D. J., Kreig, N. R., Staley, J. T. (Eds.). *Bergey's Manual of Systemic Bacteriology. The Proteobacteria, Part B. The Gammaproteobacteria*, 2nd Edn. Vol. 2 Springer, New York, pp 494-546.
10. Fallah, H. S., Asgharpour, F., Naderian, Z. and Moulana, Z. (2013). Isolation and determination of antibiotic resistance patterns in non-typical *Salmonella* species isolated from chicken. *International Journal of Enteric Pathogens*, 1(1):17-21.
11. Foley, S. L. and Lynne, A. M. (2008). Food animal-associated *Salmonella* challenges: pathogenicity and antimicrobial resistance. *Journal of Animal Science*, 86, 173-187.
12. Heinitz, M. L., Ruble, R. D., Wagner, D. E. and Tatini, S. R. (2000). Incidence of *Salmonella* in fish and seafood. *Journal of Food Protection*, 63, 579-592.
13. Huss, H. H., Reiley, A. and Embarek, P. K. B. (2000). Prevention and control of hazards in sea foods. *Food Control*, 11, 149-156.
14. ICMSF (1986). *Microorganisms in Food II. Sampling for Microbiological Analysis: Principles and Specific Applications*, 2nd Ed. Buffalo, NY: University of Toronto press.
15. Khan, A. A., Cheng, C., Van, K. T., Summage-West, C., Nawaz, M. S. and Khan, S. A. (2006). Characterization of Class 1 Integron resistance gene cassettes in *Salmonella enteric* serovars Oslo and Bareilly from imported seafood. *Journal of Antimicrobial Chemotherapy*, 58, 1308-1310.
16. Mafu, N. C., Pironcheva, G. and Okoh, A. I. (2009). Genetic diversity and *in vitro* antibiotic susceptibility sources in the Eastern Cape province of South Africa. *African Journal of Biotechnology*, 8, 1263-1269.
17. Majowicz, S. E., Musto, J., Scallan, E., Angulo, F. J., Kirk, M., O'Brien, S. J., Jones, F. J., Fazil, A. and Hoekstra, R. M. (2010). The Global Burden of Non-typhoidal *Salmonella* Gastroenteritis. *Clinical Infectious Diseases*, 50(6-15): 882-889.
18. Martinez-Urtaza, J., Saco, M., Hernandez-Cordova, G., Lozana, A., Garcia-Martin, O and Espinosa, J. (2003). Identification of *Salmonella* serovars isolated from live molluscan shellfish and their significance in the marine environment. *Journal of Food Protection*, 66(2): 226-232.
19. Minami, A., Chaicumpa, W., Chongsa-Nguan, M., Samosornsuk, S., Monden, S., Takeshi, K. (2010). Prevalence of foodborne pathogens in open markets and supermarkets in Thailand. *Food Control*, 21, 221-226.
20. Muli, R., Giljanovi, S., Ropac, D. and Katalini, V. (2004). Some epidemiologic characteristics of foodborne intoxications in Croatia during the 1992-2001 period. *Acta medica Croatica: casopis Hrvatske akademije medicinskih znanosti*, 58(5): 421

21. Newell, D. G., Koopman, M., Verhoef, L., Duizer, E., Aidara-Kane, A. and Sprong, H. (2010). Food-borne diseases-The challenges of 20 years ago still persist while new ones continue to emerge. *International Journal of Food Microbiology*, 139, S3-S15.
22. Omenwa, V. C., Ansa, E. J., Uka, A. and George, O. S. (2011). Microbiological Quality of Raw Processed Farm-Reared Periwinkles from Brackish water earthen Pond Bugumu, Nigeria. *African Journal of Food, Agriculture, Nutrition and Development*, 11(2):4623-4631.
23. Panisello, P.J., Rooney, R., Quantick, P.C. and Stanwell-Smith, R. (2000). Application of food-borne disease outbreak data in the development and maintenance of HACCP systems. *International Journal of Food Microbiology*, 59: 221-234.
24. Pui, C. F., Wong, W. C., Chai, L. C., Tunung, R., Jeyaletchumi, P., Noor Hidayah, M. S., Ubong, A., Farinazleen, M. G., Cheah, Y. K. and Son, R. (2011). *Salmonella*: A foodborne pathogen. *International Food and Research Journal*, 18, 465-473.
25. Robson, A. (2006). Shellfish view of Omega-3 and sustainable fisheries. *Nature*, 444, 1002.
26. Ruppert, E. E., Fox, R. S. and Bames, R. D. (2004). *Invertebrates Zoology* (7th ed.). Denver: Dream Books Company, LLC.
27. Solomon, L., Ogugbue, C. J. and Okpokwasili, G. C. (2013). Antibiotic Resistance Profiles of Bacteria Associated with Fresh and Frozen Shrimp (*Palaemonetes* sp.) and Their Public Health Significance. *International Journal of Scientific Research in Knowledge* (IJSRK), 1(10): 448-456.
28. Tawari, C.C and Davies, O. (2009). Effectiveness of agricultural agencies in fisheries production and management in Niger Delta, Nigeria. *Ozean Journal of Applied Sciences*, 2(4).
29. USDA/ FSIS (2008). Food Safety and Inspection Service's (FSIS) Quarterly Enforcement Report of the USDA. January 1 – March 31, 2008. <http://www.fsis.usda.gov>.
30. Walsh, T. R. and Toleman, M. A. (2012). The emergence of pan-resistant Gram-negative pathogens merits a rapid global political response. *Journal of Antimicrobial Chemotherapy*, 67, 1-3.
31. Wilker, M. A., Cockeril, F. R., Craig, W. A., Dudley, M. N., Eliopoulos, G. M. and Hetch, D. W. (2006). *Performance standard for antimicrobial disk susceptibility test*. Approved standard (9th edn). Clinical Laboratory Standards Institute.