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ORIGINAL ARTICLE

EPIDEMIOLOGY AND ANTIBIOGRAM OF LISTERIA SPECIES FROM RETAIL RAW FROZEN ATLANTIC MACKEREL FISH (*SCOMBER SCOMBRUS*) IN NIGERIA.

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SUMMARY

This study was undertaken to determine the epidemiology and antibiogram of *Listeria species* isolated from raw frozen Atlantic Mackerel (*Scomber Scombrus*) fish sold in Enugu State Southeastern Nigeria. Twenty-five grams each of raw fish gut and skin were collected from 800 raw fish sold in 8 major markets randomly selected from the study area. Isolation of *Listeria* was done using half and full fraser broths, and polymyxin acriflavine lithium chloride ceftazidime aesculin mannitol (PALCAM) agar. Confirmation of the genus *Listeria* was done by a polymerase chain reaction. Identification of species was done using an analytic profile index (API) kit specific for *Listeria*. The antibiotic resistance of the isolates was determined using disc diffusion method. Out of the 800 fish samples, 38 (4.75%) were positive for *Listeria species*. Enugu North (6.50%) had significantly ($P < 0.05$) higher prevalence than Enugu East (3.0%). Five *Listeria species* were identified. *Listeria innocua* (71.1%) predominated, followed by *Listeria welshimeri* (10.5%), *Listeria ivanovii* (7.9%), *Listeria grayi* (5.3%) and *Listeria monocytogenes* (5.3%). All the isolates were susceptible to the tested antibacterial agents. The occurrence of *Listeria species* in raw frozen Atlantic mackerel fish, poses a threat, when consumed raw or lightly cooked, to people especially the immunocompromised persons whose population is increasing as a result of improvement in medical technology. Also, the possibility of cross contamination in the kitchen or food service establishments is also a concern. Since all the *Listeria species* got in this study showed 100% susceptibility to all the antimicrobials tested, therefore,

there is no fear of acquiring and transferring antibiotic resistance genes from raw frozen Atlantic mackerel fish to the general human population.

KEYWORDS: *Listeria species*, frozen Atlantic Mackerel fish, *Scomber Scombrus*, antibiogram, molecular epidemiology, API kit

Running title: *Listeria species* in raw frozen fish

INTRODUCTION

There is an increase in the consumption of seafood products due to increased consumer awareness of nutrition and food quality. Seafood is an excellent source of high-quality protein and contains lipids with high levels of unsaturated fatty acids, which have been found to reduce the risk of cardiovascular disease (Raatz et al., 2013). In addition, seafood is tender, easily digested, and a good source of many important vitamins and minerals (Ghanbari et al., 2013). Unfortunately, seafoods can also serve a source of foodborne pathogens like *Salmonella species*, pathogenic *Escherichia coli*, *Listeria species*, and so on thereby resulting in foodborne diseases. Foodborne diseases comprise a wide spectrum of illnesses and are a rising public health concern worldwide (Ryu et al., 2013). Although, salmonellosis and campylobacteriosis have the highest occurrence rates in humans (Yun et al., 2016; EFSA, 2018), listeriosis has been recognized as one of the most serious foodborne diseases worldwide because of its severity of manifestations and high case fatality rates in both animals and humans (EFSA and ECDC, 2018). Since the recognition of animal and human listeriosis as infections caused by *Listeria monocytogenes* and less frequently by other *Listeria species*, and despite efforts made by different regulatory agencies throughout the world, listeriosis still remains a serious but neglected

public health problem worldwide (EFSA and ECDC, 2018). The genus *Listeria* is made up of small rod-shaped Gram-positive bacteria. It currently consists of 17 recognized species (*Listeria monocytogenes*, *Listeria seeligeri*, *Listeria ivanovii*, *Listeria welshimeri*, *Listeria marthii*, *Listeria innocua*, *Listeria grayi*, *Listeria fleischmannii*, *Listeria floridensis*, *Listeria aquatica*, *Listeria newyorkensis*, *Listeria cornellensis*, *Listeria rocourtiae*, *Listeria weihenstephanensis*, *Listeria grandensis*, *Listeria riparia* and *Listeria booriae*) (Abay et al., 2019). Out of these, only *Listeria. monocytogenes* has been authentically reported by many researchers as a human and animal pathogen (Orsi and Weidmann, 2016). It is an important foodborne pathogen and the third leading cause of foodborne deaths due to microbial origin in the USA (Scallan et al., 2011). Listeriosis is characterized by suppurative meningoencephalitis (that manifests as circling, excessive salivation, and unilateral facial paralysis) with brainstem micro-abscesses (Oevermann et al., 2010a). Other clinical manifestations are late-term abortion, neonatal septicaemia, and in rare cases, mastitis, gastroenteritis, and keratoconjunctivitis/uveitis (Overmann et al., 2010b, Fairley et al., 2012, Matto et al., 2023) *L. monocytogenes* has been reported to cause pregnancy losses in healthy women, and septicaemia or central nervous system disease in immunocompromised, newborn or elderly people in Nigeria (Onyemulukwe et al., 1982). *L. ivanovii* has been identified and reported to be pathogenic to animals mainly sheep and cattle. It has different clinical manifestations in ruminants but the neurological form is the most commonly seen (Oevermann et al., 2010b, Rissi et al., 2010b, Konradt et al., 2017), especially in South America- Brazil, Argentina and Uruguay (Rissi et al., 2010a, Konradt et al.,

2017). However, reports of rare cases of human listeriosis by *Listeria ivanovii* and *L. seeligeri*, in different continents, including Europe and Asia, have been published (Snapir et al. 2006; Guillet et al. 2010; Beye et al., 2016). *Listeria innocua* was reported to harbour antimicrobial resistance genes and thus has the ability to transfer such genes to other microorganisms including the pathogenic ones (Morvan et al., 2010). Generally, the presence of any *Listeria species* in food is an indicator of poor hygiene (Rocourt et al. 2000) and a potential habitat for the pathogenic *Listeria species*. Also, with the current increasing resistance of microorganisms to antimicrobials, even the nonpathogenic *Listeria species* can serve as a source of transfer of resistance genes to the pathogenic *species* as well as to other microorganisms. *Listeria species* have been isolated frequently from fish and fish products from different parts of the world (Basti et al. 2006; Parihar et al. 2008; Wan Norhana et al. 2010). Major outbreaks of listeriosis have been associated with the consumption of foods of animal origin (Rocourt et al. 2000), as well as sea foods such as shrimp, mussels and undercooked fish (Wan Norhana et al. 2010). Despite worldwide reports of increasing rate of human listeriosis outbreaks (Wang et al., 2013; Desai et al., 2019; Hanson et al., 2019; Halbedel et al., 2019; Marus, 2019), the only two human cases of listeriosis in Nigeria were reported as far back as 1982 and 1983 (Onyemelukwe et al., 1982 and 1983). Recently, none has been reported in Nigeria even with increasing reports of the isolation of *Listeria* organisms from food animals, foods of animal origin, including fish consumed in Nigeria (Nwaiwu, 2016; Usman et al., 2016; Amusan et al., 2017; Okorie-Kanu et al., 2020). Since fish and fishery products may be a vehicle for *Listeria species*, epidemiological studies are of importance

in the prevention of listeriosis outbreaks that may be caused by raw seafoods in Nigeria. Therefore, the aim of this study was to isolate *Listeria species* from raw frozen fish (*Scomber scombrus*) imported and sold at markets in Enugu State, Southeastern Nigeria, and to determine the antimicrobial susceptibility profile of the isolates.

MATERIALS AND METHODS

Study area

This study was conducted in Enugu State, Southeast Nigeria. The State comprises three agricultural zones and 17 Local Government Areas. Enugu State is located at latitudes 5°56' North and 7° 55' North and longitudes 6° 53' East and 7°55' East. Imported frozen fish is one of the sources of animal proteins consumed by the people of Enugu State.

Study population, design, and duration

The study population comprised raw frozen Atlantic markerel fish (*Scomber scombrus*) sold at the general markets in Enugu State. Meat and fish consumption form part of the food habit of the people. This fish which is widely consumed by the people in Enugu State (Kings, 2018) were observed, during the course of this study, to be imported from Japan, Mauritania, Chile, USA, and South Africa. A cross-sectional study design which lasted for 6 months (January- June 2021) was used for this study.

Sampling procedure

Two (Enugu North and Enugu East) of the

three agricultural zones in Enugu State were purposively selected because of the high sale capacity of Atlantic Mackerel fish in these areas as a result of the concentration of government parastatals and tertiary institutions, that attracted a lot of people from different areas to these selected agricultural zones. Two (2) Local Government Areas (LGAs) where these government parastatals and tertiary institutions are located were purposively chosen from each selected agricultural zone making a total of 4 LGAs. Two (2) major markets in each selected LGA were visited and 100 raw frozen fish samples each were collected from each market using Simple Random Sample (SRS) technique. In all, a total of 800 raw whole frozen fish samples were collected for the study.

Isolation and Identification of *Listeria* species from samples

Listeria was isolated from the raw frozen fish samples using a two-stage enrichment procedure outlined by the International Organization of Standardization (ISO 11290- 1) (2004). Briefly, 25g representative portion from each sample was weighed out and introduced aseptically into a sterile bag containing 225ml of pre-enrichment supplemented half-Fraser broth (HFB) (Oxoid, Basingstoke, UK) and pulverized for 2 minutes using a stomacher. The broth was then transferred into a sterile 250ml measuring cylinder covered with foil and incubated at 37°C for 24 h in ambient air. Then, 0.1ml of the HFB culture was inoculated into 10 ml enrichment full-Fraser broth (FFB) (Oxoid, Basingstoke, UK) with full-Fraser supplement, and incubated at 37°C for 48 h in ambient air. A loopful of the FFB culture was streaked onto polymyxin acriflavin lithium chloride ceftazidime aesculin mannitol (PALCAM) agar plates (Oxoid, Basingstoke, UK), and

incubated at 37°C for 48 h. The plates were observed for suspected *Listeria species* colonies at 24 hours and 48 hours of incubation. Suspected *Listeria* isolates (small-sized grayish-green colonies) were purified by sub-culturing onto fresh supplemented PALCAM agar plates and incubated at 37°C for 48 h. Pure cultures of the isolates were then inoculated on supplemented PALCAM agar slants, incubated at 37°C for 48 h, and then stored in a refrigerator at 4°C as stock bacterial cultures until needed for further analysis.

Speciation of *Listeria* isolates

Phenotypic characterization of the isolates to species level was done by subjecting them to Gram staining and various biochemical tests such as catalase and haemolysis tests, as well as arylamidase (3,3- diindolyl-methane test), esculin hydrolysis, presence of α -mannosidase, and fermentation of D-arabitol, D-xylose, L-rhamnose, α -methyl-Dglucoside, D-ribose, glucose-1-phosphate, and D-tagatose, using analytic profile index (API) *Listeria* kit (BioMerieux S.A., Marcy- l'Etoile, France) in accordance with the manufacturer's instructions.

Antibacterial Susceptibility Testing (AST)

Antibacterial susceptibility of the 38 *Listeria species* isolates was determined by the disc diffusion method using discs impregnated with 10 different antibacterial agents belonging to seven classes: β -lactam – penicillin (PEN, 10 units), amoxicillin (AMX 10 μ g), ampicillin (AMP, 2 μ g) and cephalothin (CEF, 30 μ g); aminoglycosides – gentamicin (GEN, 30 μ g)

and streptomycin (STM, 20 μ g); glycopeptides – vancomycin (VAN, 30 μ g); tetracyclines – tetracycline (TET, 30 μ g); fluoroquinolones – ciprofloxacin (CIP, 30 μ g); macrolides – erythromycin (ERY, 15 μ g) and folate pathway inhibitors – sulphamethoxazole/trimethoprim (SXT, 25 μ g). *L. monocytogenes* American Type Culture Collection (ATCC) 13932, *Streptococcus pneumoniae* ATCC 49619 and *Staphylococcus aureus* ATCC 29213 were used as reference strains. Results of the AST were interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2021) recommendations for *Listeria monocytogenes* and Clinical and Laboratory Standards Institute (CLSI, 2018) recommendations for *S. pneumoniae* (for the results of CEF) and staphylococci (for the results of PEN, AMX, VAN, TET, GEN, and CIP), respectively.

Statistical Analysis

Statistical analysis (chi-square) was done with

SPSS software version 23 to compare the association between the occurrence of *Listeria species* and various agricultural zones. The results generated were presented using descriptive statistics in tables.

RESULTS

Prevalence of *Listeria species* in raw frozen fish samples

Out of the 800 raw frozen fish samples processed, 38 were contaminated with *Listeria species*, thus giving an overall prevalence of 4.75%. Enugu North agricultural zone recorded a prevalence of 6.5% (26 out of 400) while Enugu East agricultural zone demonstrated a prevalence of 3.0% (12 out of 400). Enugu North showed significantly ($P=0.02$; $\chi^2=5.415$) higher occurrence of *Listeria* than Enugu East (Table 1).

TABLE I: Rates of *Listeria species* isolation from raw frozen fish samples

<i>Location</i>	<i>No. of samples</i>	<i>No. positive</i>	<i>Prevalence</i>	<i>95%CI</i>
Enugu East	400	12	3.0%	1.3- 4.7
Enugu North	400	26	6.5%	4.1- 8.9
Enugu State (Total)	800	38	4.75%	3.28 - 6.23

$\chi^2 = 5.415$; $P= 0.02$; Odds Ratio = 2.248; CI= Confidence Interval

Distribution of *Listeria species* isolated from raw frozen fish.

Five species of *Listeria* were isolated with *Listeria innocua* (71.1%) being the predominant species, followed by *Listeria welshimeri* (10.5%), *Listeria ivanovii* (7.9%), *Listeria grayi* (5.3%) and *Listeria monocytogenes* (5.3%) (Table 2).

TABLE II: Distribution of *Listeria species* obtained from raw frozen fish (*Scomber scombrus*)

<i>Senatorial zone</i>	<i>Listeria innocua</i>	<i>Listeria welshimeri</i>	<i>Listeria ivanovii</i>	<i>Listeria grayi</i>	<i>Listeria monocytogenes</i>	<i>Total</i>
Enugu East	9	1	1	1	0	12
Enugu North	18	3	2	1	2	26
Total	27	4	3	2	2	38
Percentage	71.1%	10.5%	7.9%	5.3%	5.3%	100%

Antibiotic Susceptibility Profile of *Listeria* isolates

All the *Listeria* isolates in this study were susceptible to the tested antibacterial agents.

DISCUSSION

Although there have been many reports of contamination of foods of animal origin and their products as well as their processing environments with *Listeria monocytogenes* and other *Listeria species*, there have been very limited studies on the occurrence and antibiogram of *Listeria species* in raw frozen Atlantic mackerel fish (*Scomber scombrus*) imported into Nigeria. The 4.75% isolation prevalence in this study, indicates that a low percentage of raw frozen Atlantic Mackerel (*Scomber scombrus*) marketed in Enugu State

Southeastern Nigeria is contaminated by *Listeria*. This finding of *Listeria* in raw frozen Atlantic fish may not be surprising because these organisms are halotropic and psychotropic (Bucur *et al.*, 2018). Possible sources of *Listeria* in the fish include: their habitat (ocean) which possibly was contaminated by anthropogenic/agricultural wastes, fish handlers both at the source of importation and retail point, cross-contamination from formites (storage tanks/freezers/crates in ships) and flies (vectors of microorganisms) that perched on the fish at the retail point. Although the prevalence of *Listeria* in this study is low, these fishes constitute a reservoir for the dissemination of *Listeria* into the human population. Cross-contamination of handlers (sellers and potential buyers) of these fishes, other food items during

storage in the refrigerator, and kitchen environment during cooking preparation, are routes through which these organisms could enter the human population. The 4.75% prevalence in this study is lower than 25.4% *Listeria* prevalence in 53 Blue Whiting fish randomly sampled from markets in Lagos State Southwestern Nigeria. (Amusan *et al.*, 2017). It could also be attributed to the measure put in place during the sale of this fish, as they were packed and wrapped in airtight containers to prevent them from defrosting. This indirectly prevented environmental contamination and deterioration of the frozen fish, thereby reducing microbial contamination. The isolation of five species (*L. innocua*, *L. welshimeri*, *L. ivanovii*, *L. grayi* and *L. monocytogenes*) indicates that a diversity of *Listeria* species contaminates raw frozen fishes imported and sold in Nigeria. Amusan *et al.* (2017) similarly isolated these 5 *Listeria* species from raw fish, but the prevalence of the organisms varied with that of this study. The fishes in this study were imported from various countries (Mauritania, USA, Japan, Chile, South Africa) where all the 5 *Listeria* species and more have also been reported in fishes (Jinneman *et al.*, 1999; Yamazaki *et al.*, 2000; Wu *et al.*, 2015). Therefore, it is possible that these organisms were imported from the countries where they were captured. It could also mean contamination from the storage, sellers and/or buyers (who touched the fishes with bare hands, thereby contaminating them), since *Listeria* species has been reported from different food items, animals and humans (Ikeh *et al.*, 2010; Ndahi *et al.*, 2013; Okorie-Kanu *et al.*, 2020) in Nigeria. The prevalence of *Listeria monocytogenes* obtained from this study is 5.3% (2 out of 38). This is significant especially because of the stringent “zero tolerance” policy for foods contaminated by *Listeria monocytogenes* (Farber *et al.*, 2021). This organism could pose a serious public health threat

to consumers especially the population at risk of listeriosis (immunocompromised persons like the pregnant women, children and elderly) as well as the general public. Surprisingly, a considerable percentage of Nigerians consume fish as a relatively cheaper source of quality protein readily available to the poor people of Nigeria and other developing countries (Akinbode and Dipeolu, 2012). The 5.3 % prevalence of *Listeria monocytogenes* in this study is similar to 6.2% *L. monocytogenes* prevalence reported by Amusan *et al.* (2017) in raw fish in Lagos, Nigeria.. However, higher prevalence rates of 25% and 12.88% were reported in smoked and fried fish from Sokoto and Kwara States, respectively (Salihu *et al.*, 2008; Adeshina *et al.*, 2017). An earlier report has documented *Listeria* species other than *Listeria monocytogenes* in fish in the tropical area (Manoj *et al.*, 1991). Our study detected *Listeria innocua* (71.1%) as the most predominant *Listeria* species in frozen fish sold in Enugu State, followed by *Listeria welshimeri* (10.5%), *Listeria ivanovii* (7.9%) and then *Listeria grayi* (5.3%). Other researchers have also reported *Listeria innocua* as the dominant species in fish (Ammar *et al.*, 2014; Jamali *et al.*, 2015). The reason could be because *Listeria monocytogenes* and *Listeria innocua* share the same ecological niche, where the isolation of *Listeria innocua* and other non-pathogenic species in fish and fish products serve as indicators of the presence of *Listeria monocytogenes* (Batt *et al.*, 2014). The presence of *Listeria innocua* in frozen fish studied could also mean a potential source of infection for immunocompromised persons considering the reports of Perrin *et al.* (2003) and Favaro *et al.* (2014) who documented fatal cases of bacteraemia and meningitis due to *Listeria*

innocua in humans. Moreover, cases of human listeriosis (manifested as meningitis) associated with food-borne *Listeria seeligeri*, *Listeria ivanovii* and *Listeria welshimeri* have also been reported (Rocourt *et al.*, 1986; Gasanov *et al.*, 2005; Snapir *et al.*, 2006; Guillet *et al.*, 2010; Beye *et al.*, 2016). Therefore, the presence of *Listeria species* other than *Listeria monocytogenes* should always be considered a public health threat. Also, the risk associated with the isolation of *Listeria species* especially *Listeria innocua* is the increased potential of the organism to transfer resistance genes to other bacteria species capable of causing human diseases (Gomez *et al.*, 2014). Many studies have reported increasing antibiotic resistance in *Listeria species* albeit low rates (Charpentier *et al.*, 1999; Li *et al.*, 2007). In this study, 100% susceptibility to all the tested antibacterial agents was exhibited by the *Listeria* isolates. The 100% susceptibility recorded could be attributed to the fact that these Atlantic Mackerel frozen fish examined are found in the Atlantic Ocean, their natural habitat. Additionally, Atlantic mackerel are feral marine fishes and therefore, the *Listeria* isolates may not have been previously exposed to antimicrobials that could have led to selective pressure. This supports the report of Wang *et al.* (2017) who isolated *Listeria species* from wild rodents and found that the *Listeria species* were sensitive to majority of the antimicrobials tested. Indiscriminate use of antimicrobial agents in human and veterinary medicine causes the development of resistance by microorganisms (Chang *et al.*, 2014; Jamali *et al.*, 2015). However, the level and type of resistance are affected by regional differences (Bertrand *et al.*, 2005). Considering the critical importance of ampicillin/penicillin G and gentamicin as reference therapy for human listeriosis, and sulphamethoxazole/trimethoprim, vancomycin, and erythromycin as second-choice

drug for managing listeriosis in pregnant women (Hof, 2004), it is desirable that this favourable situation (lack of resistance observed in this study) is preserved through the establishment of antimicrobial stewardship programmes emphasizing on personal hygiene of frozen fish sellers/buyers. The 100% susceptibility recorded in this work is consistent with the findings of Salamoura *et al.* (2008) who also did not observe resistance among *Listeria monocytogenes* and other *Listeria species* isolated from marine fish. But it disagrees with the work of Adebayo-Tayo *et al.* (2012) who reported varying degrees of resistance to chloramphenicol, amoxicillin, oxacillin, trimethoprim, streptomycin and ciprofloxacin in frozen fish in Akwa-Ibom, Nigeria.

CONCLUSION

This study has shown that a low percentage (4.75%) of imported frozen raw Atlantic mackerel sold in Enugu state Southeast Nigeria is colonized by *Listeria*. A diversity of *Listeria* species including *L. monocytogenes* and other *Listeria* species contaminates imported frozen fish in the study area. Thus, individuals who handle and consume these fishes raw/half-cooked, are at risk of food-borne listeriosis. In addition, the possibility of cross contamination in the processing plant, kitchen or food service establishments is also a concern.

RECOMMENDATIONS

We recommend that thorough microbiological examination of raw frozen fish imported into the country should always be done by NAFDAC in conjunction with Veterinary officers so as to detect zoonotic microorganisms of public health

importance. Also, standard of hygiene should be improved at the point of sale to ensure food safety.

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CONFLICTS OF INTERESTS

The authors declare that they have no conflicts of interest.

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