Isolation and Identification of *Listeria monocytogenes* in Fresh Croaker (*Pseudotolithus senegalensis*)

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**ABSTRACT**

*Listeria monocytogenes* is an important foodborne pathogen. Food products contaminated with this bacterium causes listeriosis which is a disease with high mortality and hospitalization rate. It is a known threat to food safety due to its persistence in food processing environment and its ability to survive and grow at several environmental conditions. In this study, a total of 108 fresh croaker samples were collected from retail outlets in Lagos, Nigeria. The isolation of *L. monocytogenes* involved the use of culture methods based on selective enrichment and plating. Isolates were identified by colony morphology, sugar fermentation and haemolytic properties. Genotypic identification of *L. monocytogenes* was performed using PCR incorporating 16S rRNA followed by DNA sequence analysis. Eight samples (7.4%) were positive for *L. monocytogenes*. The sequence and phylogenetic analysis based on the 16S rRNA showed that fresh croaker *L. monocytogenes* isolates were grouped into two different clusters when compared with reference *L. monocytogenes* indicating that they belong to different ancestors. This study revealed the presence of *L. monocytogenes* in fresh croaker in low concentration. However, vulnerable populations are still at risk if these products are consumed raw or lightly cooked.

**Keywords:** foodborne pathogen, PCR, phylogenetic analysis.

**1. INTRODUCTION**

Croaker (*Pseudotolithus senegalensis*) is a marine fish that is widely distributed along the Atlantic Coast of Tropical West Africa (Edwards *et al*., 2001). They are of high market value and are usually caught by artisanal and industrial fisheries (Adejonwo, 2016; Jim-Saiki *et al*., 2016). *Listeria* species are short Gram positive rods with rounded ends, occurring singly or in short chains and occasionally appearing filamentous (PHE, 2014). They are catalase positive, oxidase negative and non-sporulating motile bacteria (Bayoub *et al*., 2010). The genus *Listeria* harbors seventeen species having *Listeria monocytogenes* and *L. ivanovii* as its pathogenic species (Orsi and Wiedmann, 2016). *Listeria monocytogenes*, an important foodborne pathogen is widely dispersed in the environment. It is found in soil, water and plant materials (Leong *et al*., 2016). It has also been isolated from raw and unpasteurized milk, cheese, ice cream, raw vegetables, fermented meats and sausages, raw and cooked poultry products, raw meats, raw and smoked seafoods (Nakari *et al*., 2014; Jami *et al*., 2014).
Listeria monocytogenes is a threat to food safety because it can grow at refrigerated temperature (4 ºC - 10 ºC), wide pH range and high salt concentration (Bortolussi, 2008). They can contaminate processing plants and retail environments in which ready-to-eat foods are produced and served (Jarvis et al., 2016). Listerosis caused by Listeria species is recognized as a foodborne disease and consumption of ready-to-eat foods is the main route of transmission to humans (Miya et al., 2010; Elbashir et al., 2018). It is a disease that has emerged along with changing eating habits and large-scale industrial food processing. It leads to losses of billions of dollars yearly, recalls of contaminated foods and patients medical treatment expenses (Camargo et al., 2016). Several selective enrichment and plating media have been developed and used for the isolation and detection of Listeria spp. in food and environmental samples. The requirements of various regulatory agencies are that the isolation methods must be able to detect one Listeria organism per 25 g of food. To achieve this sensitivity, enrichment methods are required to allow the organism to grow and reach a detectable level before plating onto selective media and confirmation of cultures (Law et al., 2015). The use of selective agar to identify Listeria spp. is generally quite accurate, confirmation by other means should always be performed before any conclusions are drawn. Common methods of confirmation of Listeria spp. include confirmation by PCR, API kits, Microbact and sequencing (Leong, 2017). According to Nwaiwu (2015), reports on the occurrence of Listeria spp. in Nigeria are based on classical serotyping, biochemical tests and dark colouration of media due to hydrolysis of aesculin. Thus there is a need to utilize the current polymerase chain reaction (PCR) based on molecular techniques to characterize L. monocytogenes from fresh croaker so that accurate information on existing Listeria strains and sources of infection can be established.

2. MATERIALS AND METHODS

2.1. Sample Collection

A total of 108 fresh croaker samples were purchased from five retail outlets (Badagry, Iyana Ipaja, Liverpool, Makoko and Mushin) in Lagos State, Nigeria. The samples were collected in sterile specimen bags under chilled conditions and taken to the Microbiology laboratory at the Nigerian Institute for Oceanography and Marine Research, Victoria Island for analysis.

2.2. Isolation and identification of Listeria monocytogenes

The isolation of Listeria monocytogenes was carried out using a single enrichment broth and plating method. For each sample, 25 g was added to 225 mL of one-broth Listeria and stomached for 30 seconds. It was incubated at 30 ºC for 24 hours. After 24 hours enrichment, the broth cultures were agitated and a loopful was inoculated onto Brilliance Listeria agar. This was incubated appropriately (37 ºC for 24 – 48 hours). The plates were examined for blue-green colonies with white halos, 3 suspected colonies were subcultured on Tryptone Soya Agar (TSA, Oxoid) and incubated at 37 ºC for 24 hours. Phenotypic characterization such as Gram’s staining, Catalase and motility tests were carried out according to the methods of Hitchins and Jinneman, 2011. Oxoid Listeria Latex Agglutination Test, Oxoid Biochemical Identification System (O.B.I.S.) and MICROBACT Listeria 12L system were also used for phenotypic characterisation.

2.3. Genotypic Characterisation
2.3.1. DNA Isolation
A single colony of *L. monocytogenes* was grown overnight on TSA at 37 °C. One colony from the culture was inoculated into 5 mL of Tryptone Soya Broth and incubated overnight at 37 °C. The broth culture of 2.5 mL was centrifuged and the pellet washed in 1 mL of distilled water and resuspended in an Eppendorf tube containing 400 μl of phosphate buffer saline (PBS). Deoxyribonucleic acid (DNA) was extracted using Qiagen DNA extraction kit according to the manufacturer’s instruction.

2.3.2. Amplification of 16S rRNA region of Listeria by Polymerase Chain Reaction (PCR)
The universal primers for *Listeria* (F: 5′CAGCAGCCGCGGTAATAC 3′ and R: 5′ CTCCATAAGGGTGACCCT 3′) were used for the amplification of the 16S rRNA region of the isolates using C1000 Touch Thermal cycler (BioRad, USA). The PCR mixture consisted of 2.5 μL 10×PCR buffer, 2.0 μL dNTP (deoxyribonucleoside triphosphate), 0.25 μL each of the forward and reverse primers, 0.25 μL AmphiTaq DNA polymerase (TransGen Biotech, China), 14.75 μL sterile distilled water and 5 μL of appropriate DNA preparation. A reaction mixture with no DNA template was used as a negative control. The cycling conditions were denaturation at 95 °C for 3 minutes followed by 35 cycles of amplification (each cycle consisted of denaturation at 94 °C for 1 minute, annealing at 60 °C for 2 minutes and elongation at 72 °C for 1 minute). A final extension was performed for 10 minutes at 72 °C. The amplified products (5 μL) were analysed by 1.5% agarose gel electrophoresis and ethidium bromide staining. The DNA bands were observed under an ultraviolet (UV) transilluminator (Park et al., 2012). The amplified products (20 μL) were sent to GATC Biotech, Germany for sequencing. The sequences obtained were blasted and compared with similar sequences in the Gene Bank (NCBI, USA).

3. RESULTS
The phenotypic characterisation of *L. monocytogenes* isolated from fresh croaker showed that all isolates were Gram positive short rods, catalase positive, oxidase negative, motile and gave positive reaction to *Listeria* test kit. The O.B.I.S. mono test gave a negative result for *L. monocytogenes*. The Microbact 12L system was also used for the full identification of *L. monocytogenes* (Table 1). The occurrence of *L. monocytogenes* in fresh croaker samples are shown in Table 2. Eight isolates were characterised by using 16S rRNA primers specific for *Listeria* species, out of which 6 isolates showed amplification. This is shown in plate 1. Three isolates were fully identified as *L. monocytogenes* while the remaining 3 had *Listeria* spp. as its identity. The sequences of three isolates that were fully identified from this study have been deposited in the Gene Bank database under accession numbers: MG670095, MG670096 and MG670097 respectively. The evolutionary relationships between the *L. monocytogenes* from this study and those reported in GenBank were evaluated using the NJ method described by Kumar et al. (2016) conducted in MEGA 7 (Figure 1).
TABLE 1: Phenotypic Characterisation of *Listeria monocytogenes*

| Sample Code | Gram Reaction | Shape | Catalase | Oxidase | Latex Agglutination | Motility | OBIS | Arabinol | Rhamnose | Trehalose | Haemolysis | Probable Organism |
|-------------|---------------|-------|----------|---------|----------------------|----------|------|----------|----------|-----------|------------|-------------------|
| FC1         | +             | SR    | +        | -       | +                    | -        | +    | +        | +        | +         | +          | *L. monocytogenes* |
| FC2         | +             | SR    | +        | -       | +                    | -        | +    | +        | +        | +         | +          | *L. monocytogenes* |
| FC3         | +             | SR    | +        | -       | +                    | -        | +    | +        | +        | +         | +          | *L. monocytogenes* |
| FC4         | +             | SR    | +        | -       | +                    | -        | +    | +        | +        | +         | +          | *L. monocytogenes* |
| FC5         | +             | SR    | +        | -       | +                    | -        | +    | +        | +        | +         | +          | *L. monocytogenes* |
| FC6         | +             | SR    | +        | -       | +                    | -        | +    | +        | +        | +         | +          | *L. monocytogenes* |
| FC7         | +             | SR    | +        | -       | +                    | -        | +    | +        | +        | +         | +          | *L. monocytogenes* |
| FC8         | +             | SR    | +        | -       | +                    | -        | +    | +        | +        | +         | +          | *L. monocytogenes* |

Key: + = Positive; - = Negative; SR = Short Rod; FC= Fresh Croaker; OBIS = Oxoid Biochemical Identification System
Table 2: Occurrence of *L. monocytogenes* in Fresh Croaker

| Retail Outlets | Number of samples Analysed | Number of samples positive for *L. monocytogenes* [n (%)] |
|---------------|-----------------------------|----------------------------------------------------------|
| Badagry       | 15                          | 0 (0.0)                                                  |
| Iyana Ipaja   | 19                          | 0 (0.0)                                                  |
| Liverpool     | 30                          | 6 (20.0)                                                 |
| Makoko        | 21                          | 2 (9.5)                                                  |
| Mushin        | 23                          | 0 (0.0)                                                  |
| **Total**     | **108**                     | **8 (7.4)**                                              |

**PLATE 1:** Agarose gel showing Polymerase Chain Reaction amplified product of 938 bp for 16S rRNA specific primer for *Listeria* spp.

Lane M: Molecular Weight Marker, Lanes 1, 4-5, 7-8, 10: *L. monocytogenes*, Lane 6: control reaction
4. DISCUSSION

Listeria monocytogenes is a foodborne pathogen that contaminates food-processing environments. It persists as biofilms on equipment, utensils, floors and drains, ultimately reaching final products by cross-contamination. Although, the estimated incidence of listeriosis is lower than other enteric illnesses, infections caused by L. monocytogenes can lead to hospitalizations and mortalities (Camargo, 2017). The Oxoid Novel Enrichment (ONE) broth used in this study was able to detect the presence of Listeria spp. This agreed with the work of Upham et al. (2010), who carried out a study on comparison of UVM, Palcam and ONE broth as primary enrichment broths for Listeria spp. They observed that ONE broth was preferable to...
UVM and Palcam broths to facilitate detection of *Listeria* spp. The use of selective agar in *Listeria* spp. isolation gives an initial result that is presumptively positive, but confirmation of the isolates is needed as false positives can and do occur (Leong, 2017). In this study, Brilliance Listeria Agar (BLA) was used and it differentiated between *L. monocytogenes* and other *Listeria* species by the presence of a halo it produced on the plate. This is in agreement with the work of Leong (2017) who also had similar results when chromogenic agar was used. The identification of *Listeria* species by biochemical methods is a strenuous process, involving primary isolation with selective and enrichment media, followed by Gram stain and multiple biochemical tests. Thus the incorporation of various biochemical procedures into a single testing platform has streamlined the diagnostic process for *Listeria* species (Bille et al., 1992). The Microbact 12L used in this study was able to identify *L. monocytogenes*. However, biochemical testing of *Listeria* species remains costly and time consuming (taking up to 6 days to finalize a result). Furthermore, as biochemical tests measure the phenotypic characteristics of *Listeria* bacteria, their performance can be influenced by external factors that affect bacterial growth and metabolic mechanisms (Liu, 2006). This study revealed that all isolates were Gram positive short rods, catalase positive, oxidase negative and non-sporulating motile bacteria. The biochemical characteristic of *Listeria* was similar to the findings of Bayoub et al. (2010) and Islam et al. (2016).

The result of the current study showed that 7.4% of fresh croaker was contaminated with *L. monocytogenes*. The isolation rate of *L. monocytogenes* in this study is comparable with the reports of Montaz and Yadollahi (2013) and Jamali et al. (2015) who reported occurrence of 7.7% and 7.6% respectively of *L. monocytogenes* in raw fish from Iran. In Nigeria, a relatively higher occurrence of 40% and 37.65% were reported in fresh fish from Nsukka and Calabar respectively (Ikeh et al., 2010; Lennox et al., 2017). The differences in the occurrence of *L. monocytogenes* in foods in different countries might be due to the differences in the composition of food items and hygiene status of food processing plants (Garedew et al., 2015). The use of 16S rRNA has become the method of choice for identifying and differentiating microorganisms when no other easily specified nucleic acid sequence uniquely defines the desired target. It is a distinct signature for a bacterial species (Gray et al., 1984; Woese, 1987). The study showed that 16S rRNA sequencing analysis approach was valuable in the identification of *L. monocytogenes*. This is in agreement with Aznar and Alarcon (2002) who also used 16S rRNA gene for identification purposes. Similarly, Soni and Dubey (2014) were able to identify 80 *Listeria* isolates from food, clinical and environmental samples based on 16S rRNA and sequence similarity. The phylogenetic tree based on 16S rRNA gene sequence clearly differentiated between the three *L. monocytogenes* isolates obtained in this study. *L. monocytogenes* MG670095 clustered with *L. monocytogenes* KR012147 which was isolated from deep sea sediment in Pacific Ocean. The other two isolates *L. monocytogenes* MG670096 and MG670097 formed a cluster showing that they have a common ancestor. Mohamed et al. (2016) suggested that the difference in the sequences of *Listeria* spp. could be as a result of sequencing artifacts.

5. CONCLUSION

The presence of *L. monocytogenes* in fresh croaker poses a risk to vulnerable populations if these products are consumed raw or lightly cooked. Therefore, there is a need for appropriate control measures so as to avoid contamination of fresh croaker and to assure the safety of the product.
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