

Detection of *Listeria monocytogenes* in Fried Fish, Processing Slab and Tools in Kwara State, Nigeria

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Abstract: This study investigated the prevalence of *Listeria monocytogenes* in fried fish, processing slab and tool in Kwara state, Nigeria. A total of 2400 fish samples, 800 slabs and 800 tool samples were collected aseptically using stratified sampling methods for *L. monocytogenes* analysis. The samples were cultured for *L. monocytogenes* using PALCAM and fraser selective agar and supplements. The isolates were characterized using both biochemical and molecular methods. The data obtained were subjected to descriptive statistics and chi-square test, $P=0.05$. The results have showed that 528 (22%), 313 (39.13%) and 195 (24.38%) of the fried fish, slab and tool samples are positive for *L. monocytogenes* respectively. Molecular characterization using PCR has shown that 25.87% of biochemically characterized isolates are identified as *L. monocytogenes*. *Listeria monocytogenes* was identified in fried fish (12.88%), slab (36.41%) and tool (26.41%) samples. There was a significant difference between the occurrence of *L. monocytogenes* in fish, slab and tools. Fried fish, processing slab and tools were heavily contaminated with *L. monocytogenes*. Processing slab and tools were identified to be predisposing factors, and therefore calls for attentions.

Keywords: *Listeria Monocytogenes*, Infection, Prevalence, Fried fish.

Nijerya'nın Kwara Eyaletinde Tüketime Sunulan Kızartılmış Balıklar ile Kullanılan İşleme Levhası ve Aletlerde *Listeria monocytogenes* Varlığının Belirlenmesi

Özet: Bu çalışmada Nijerya'nın Kwara eyaletinde kızartılmış balık, işleme levhası ve aletlerde *Listeria monocytogenes* prevalansı araştırılmıştır. *L. monocytogenes* analizleri için tabakalı örnekleme yöntemi kullanılarak aseptik olarak toplam 2400 balık örneği, 800 plaka ve 800 alet numunesi toplandı. Örnekler *L. monocytogenes* için PALCAM ve fraser selektif agar ve takviyeleri kullanılarak kültüre edildi. İzolatlar hem biyokimyasal hem de polimeraz zincir reaksiyonu (PZR) ile moleküler yöntemlerle karakterize edildi. Elde edilen veriler tanımlayıcı istatistik ve ki-kare testi ile analiz edildi. Sonuçlar, kızarmış balık işleme levhası ve alet örneklerinden sırasıyla 528 (%22), 313 (%39.13) ve 195'inin (%24.38) *L. monocytogenes* açısından pozitif olduğunu göstermiştir. Örneklerin PCR işlemi ile moleküler karakterizasyonunda, biyokimyasal olarak karakterize edilmiş izolatların %25.87'sinin *L. monocytogenes* olduğu tespit edilmiştir. Kızarmış balık (%12.88), işleme levhası (%36.41) ve aletlerden (%26.41) elde edilen izolatların *L. monocytogenes* olduğu belirlenmiştir. *L. monocytogenes* sıklığı açısından kızarmış balık, işleme levhası ve aletler arasındaki fark önemli bulunmuştur. Sonuç olarak, Nijerya'nın Ilorin şehrinde incelenen kızarmış balık, işleme levhası ve alet örneklerinin yüksek düzeyde *L. monocytogenes* ile kontamine olduğu tespit edilmiştir. İşleme levhaları ve aletleri predispozan faktörler olduğundan önem taşımaktadır.

Anahtar Kelimeler: *Listeria monocytogenes*, Enfeksiyon, Prevalans, Kızartılmış balık.

Introduction

Fish is highly nutritious and serves as major animal source of protein in developing countries like Nigeria. Fish is consumed in different forms such as fresh, smoked, fried or boiled. Fried fish also known as "ejadindin" is the most consumed processed fish in Nigeria. More so, fried fish is sold at lower price (as low as 5 naira equivalent to \$0.025) thereby accessible and affordable by many individuals. However, less attention is given to the quality and safety of the fried fish.

Listeria monocytogenes is a gram positive bacterium that can be found in a variety of foods and has ability to grow at the refrigerator temperature. It causes septicemia, central nervous system infection, intra-uterine infection, and meningitis in adults while new born babies and fetuses suffer from severe systemic infection (Hakim et al., 2015). Pregnant women infected with *L. monocytogenes* also show symptoms resembling to those of a mild influenza-like illness. Listeriosis

has been reported to be accounted for a mortality rate of about 20% to 50% (Acha and Szyfres, 2003; Chukwu, 2007; Rychli et al., 2014). During a listeriosis outbreak in 1985 in California State of United States of America, 52 deaths were reported by federal database (Eruteya et al., 2014). Between 1998 and 1999, at minimum, 14 people had been reported to have lost their life as a result of listeriosis outbreak from eating hot dogs in United States of America. In any case, about 13 people in eight states in United States of America have died from eating cantaloupe contaminated with *Listeria spp.* Miettinen (2006) reported that at least one out of six Americans is infected with *Listeria spp.* as a result of consumption of contaminated food on yearly bases. *Listeria* is accounted for about 500 death every year (Miettinen, 2006; Álvarez-Ordóñez et al., 2015). Almost all cases of human listeriosis are due to *L. monocytogenes* infection while infections due to *Listeria ivanovii* and *Listeria seeligeri* have also been described (Seeliger and Jones, 1986; Ieren et al., 2013). *Listeria monocytogenes* is psychrotrophic and halotolerant (de Simón et al., 1992). It can survive or even grow at pH values as low as 4.4 and at salt concentrations of up to 14%. As a consequence, it has been isolated from a variety of sources, including fish and fishery products (Els et al., 2004; Gudmundsdóttir et al., 2005; Karunasagar and Karunasagar, 2000; Nakamura et al., 2004; Katzav et al., 2004; Tocco et al., 2014). Considerable contamination occurs during evisceration, fish handling and packing as a result of inadequate hygiene (Shen et al., 2013).

The U.S. Food and Drug Administration (FDA) maintains a zero tolerance policy (FDA and FSIS, 2003; Kamat and Nair, 1993) while EU legislation (no. 2073/2005) imposes a zero-tolerance policy in respect to certain foods destined for high-risk consumer groups and otherwise limits these bacteria to below 100 CFU/g (Jami et al., 2014). However, there is no report of outbreak in Nigeria yet but the listeriosis cases might have been unreported due to low level of awareness, education and health care facilities available in the country. Therefore, we aimed, in this study, to investigate the presence and prevalence of *Listeria* species in fried fish, processing slab and tool in Kwara state, Nigeria.

Material and methods

Study area and sample collection: The study was carried out in Kwara State located in the North-central geopolitical zone of Nigeria. The state has sixteen (16) local government areas (LGAs) (KWSG, 2015). Fifty fried fish sellers were selected using stratified methods as described by Fagbenro and

Adebayo (2005). A total of 2400 fish samples consisting of 150 samples from 50 fried fish processors/sellers in each LGA as well as 800 slab and 800 tools samples were collected for the analysis (Swetha et al., 2012). Fish samples were collected in sterile bottles; swab and tools samples were collected by swabbing using swab stick and transported to the Department of Veterinary Microbiology and Parasitology, University of Ilorin for bacteriological study.

Isolation of *Listeria* species: For pre-enrichment one gram of tissue samples obtained from fried fish samples were incubated at 37 °C for 24 hours in universal bottles containing 9 ml of sterile half-Fraser broth and fraser supplement (Oxoid products SR0156). From the pre-enrichment culture media about 0.1 ml was transferred into 10ml of full strength Fraser broth with fraser supplements and was incubated at 37 °C for 24-48 hrs (secondary enrichment) (Rocourt et al., 2001). The culture media were then streaked on PALCAM prepared plates by using a wire loop and incubated at 37°C for 48 hrs. The wire loops were sterilized on spirit lamp for 60 seconds before usage. The plates with a black sunken centre and a black halo on a cherry-red background, following aesculin hydrolysis and mannitol fermentation were suspected to be colonies of listeria species and selected for further identification tests as adopted by (Eruteya et al., 2014). The suspected colonies were sub-cultured on PALCAM agar (Oxoid product CM0877) and incubated for 24-48 hours to obtain pure colonies.

Macroscopic examination of culture plates: The presumptive identification of *Listeria* species were based on the cultural and evidence of aesculin hydrolysis or black-halo formation on PALCAM plates. The suspected organisms were stored on glycerol slant and backup with slant of PALCAM, stored in a freezer and refrigerator respectively until use. Further test were carried out. The tests are: Gram stain reaction, Beta-haemolysis, catalase reaction, motility at room temperature, sugar or carbohydrate fermentation (Rhamnose, Xylose, Lactose, Fructose and Manitol).

DNA extraction: The *Listeria* species (isolates) were suspended in 1.5 ml of enriched fraser broths was grown on a shaker for 48 hours at 48 °C and pelleted by centrifugation at 6000 rpm (4600x g) for 5 min. The pellets were re-suspended in 520 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Fifteen microliters of 20% SDS and 3 µl of Proteinase K (20 mg/ml) were added. The mixture was incubated for 1 hour at 37 °C, then 100 µl of 5 M NaCl and 80 µL of a 10% CTAB solution in 0.7 M

NaCl were added and mixed. The suspension was incubated for 10 min at 65 °C and kept on ice for 15 min. An equal volume of chloroform: isoamyl alcohol (24:1) was added, followed by incubation on ice for 5 min and centrifugation at 7200 x g for 20 min. The aqueous phase was transferred to a new tube, isopropanol (1:0.6) was added and DNA was precipitated at -20 °C for 16 h. DNA was collected by centrifugation at 7200 x g for 10 min, washed with 500 µl of 70% ethanol, air-dried at room temperature for approximately three hours and finally dissolved in 50 µl of TE buffer.

Polymerase Chain Reaction (PCR): PCR reaction mix consisted of 2.5 µl of 5x GoTaqgreen reaction buffer, 0.75 µl of MgCl₂, 0.25 µl of 10 mM of dNTPs mix, 0.25 µl of 10 pmol each of forward and backwards haemolysis primer with forward primer =hlyA-F (target gene = hly; length = 24; primer sequence = 5'GCAGTTGCAAGCGCTTGGAGTGAA3 and amplification product = 456) and reverse primer=hlyA-R (target gene = hly; length = 24; primer sequence = 5'GCAACGTATCCTCCAGAGTGATCG 3 and amplification product = 456) and 0.06 µl of Taq DNA polymerase (8000U) (Promega, USA) made up to 10.5 µl with sterile distilled water then 2 µl DNA template. PCR carried out in a GeneAmp 9700 PCR System Thermalcycler (Applied Biosystem Inc., USA) as described by Swetha et al. (2012). The cycling condition for hlyA primer on a PCR profile were : initial denaturation 95 °C for 5 min, 35 cycles; final denaturation 94°C for 60 s; annealing 61 °C for 45 s and 72 °C for 60 s; a final extension at 72 °C for 7 mins and held chill at 4 °C (Swetha et al., 2012).

Integrity: The integrity of the amplified band for haemolysis gene fragment was checked on a 1.5% Agarose gel ran at 110V for about 1 hour to confirm amplification. Picture was taken under gel electrophoresis indicating amplification using haemolysis specific primer.

Statistical analysis: The data obtained from the experiment were subjected to descriptive statistics. The prevalence was compared using Chi-square test with the aid of SPSS statistical package version 20.

Table 1. Occurrence of *L. monocytogenes* biochemically characterized from fried fish, working slab and tools in Kwara state

Samples	No. examined	Positive	Percentage	Intensity
Fish	2400	528	22.00	0.22
Slab	800	313	39.13	0.39
Tool	800	195	24.38	0.24
Total	4000	1036	25.90	0.26

Results

Figure 1 shows the black sunken centre and a black halo on a cherry-red background, following aesculin hydrolysis and mannitol fermentation of a typical listeria characteristics. Table 1 shows the result of the fried fish, working slab and tools samples examined for *L. monocytogenes*. Out of 2400 fried fish, 800 working slab and 800 tools examined, 528 (22%), 313 (39.13%) and 195 (24.38%) were positive for *L. monocytogenes* respectively. About 25.90% of the total samples examined were infected with *L. monocytogenes*.



Figure 1. Colony morphology of *Listeria* in PALCAM agar

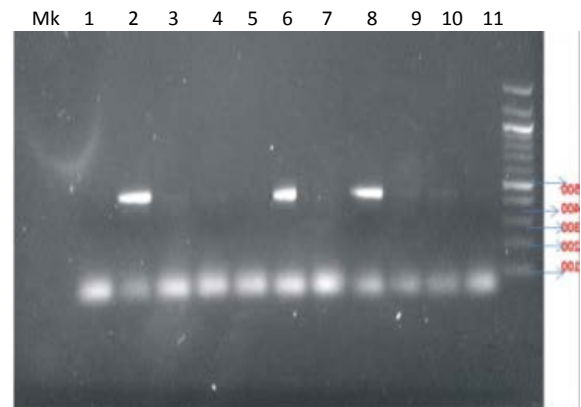


Figure 2. PCR gel of *L. monocytogenes* using hlyA primer Where: Lanes 4, 6 and 10 = positive amplified lanes for *L. Monocytogenes* Other lanes= non-amplified lanes.

The biochemically characterized isolates (n=1036) were further subjected to molecular characterization using PCR. About 25.87% were identified to be positive for *L. monocytogenes*. From fried fish samples, out of 528 isolates, 68 (12.88%) were confirmed. In working slab and tool 313 and

195 isolates were investigated molecularly and shown 129 (36.41%) and 71 (26.41%) to be *L. monocytogenes*. The intensity of infection was highest in slab (0.41) and lowest in fish (0.13) (Table 2).

Table 2. Molecular characterization of *L. monocytogenes* targeted at haemolyses gene (hlyA)

Sample	No. <i>L. spp.</i> (%)	<i>L. monocytogenes</i> (%)	Intensity	P-value
Fish	528 (22.00) ^a	68 (12.88) ^a	0.13	0.000
Slab	313 (39.13) ^b	129 (41.21) ^b	0.41	
Tool	195 (24.38) ^a	71 (36.41) ^c	0.36	
Total	1036 (25.90)	268 (25.87)	0.27	

Different superscripts in the same column refer to statistically significant (P<0.05) in prevalence

Discussion

One of the characteristics of *L. monocytogenes* is that it is ubiquitous in nature which means it is capable of surviving in various environmental conditions including refrigerated temperatures that kill several other bacteria. Presence of *L. monocytogenes* in fried fish in this study was in accordance with the fact that it has ability to survive in processing rooms/equipments even under many cleaning processes as reported by Loncarevic et al. (1996) and Jørgensen and Huss (1998). The percentage of *L. monocytogenes* recorded in fish samples in this study was similar to 10% by Dauphin et al. (2001), 18% Gudbjörnsdóttir et al. (2004), 8% by Swetha et al. (2012), 13.33% Adeshina and Adewale (2015) and 17% by Ajaiyeoba et al. (2015) but lower than 100% reported by Autio et al. (1999), 22% by Johansson et al. (1999), 47% by Fannesbech-Vogel et al. (2001), 25% by Salihu et al. (2008) and Amusan (2015). Furthermore, the findings of the present study were higher than the 1% recorded by Thimothe et al. (2004), 4% by Gudmundsdóttir et al. (2005) and 1.29% by Eruteya et al. (2014). The bacterium ability to persist in the processed food makes it a psychrotrophic pathogenic bacterium.

Listeria monocytogenes is heavily present in the processing tools (slab and cutlery) based on observation in this study. The result is in agreement with the findings of Cetinkaya et al. (2014) who reported 30% and 32% by Alvarez-Ordoriez et al. (2015) but lower than 71% recorded by Eklund et al. (1995) and 88% recorded by Ieren et al. (2013). More so, the result were higher than 15% reported by Johansson et al. (1999), 17% by Fannesbech-Vogel et al. (2001), 1% by Hoffman et al. (2003) and 3% by Pusztahelyi et al. (2016). The differences in the values might be attributed the management practices such as cleaning. The higher value in this study further suggest that the level of hygiene of

the handlers since the *L. monocytogenes* is an ubiquitous which could found in many places and of course had being isolated from several materials. Proper cleaning and hygienically process fish could contain lower *L. monocytogenes*. In the developing nations like Nigeria, the level of cleaning and hygiene practices is lower compared to developed nations where most of the compared studies were carried out, thus, could be responsible for the variations in the values. The study did not investigated the cleaning and hygiene practices by the handlers but opined it could be responsible for the differences since *L. monocytogenes* is capable of survive wide range of environmental conditions including refrigerated temperature and ability to persist in a material during cleaning.

The results have showed that there is heavy presence of *L. monocytogenes* in fried fish, processing slab and tools. The results further suggest that processing slab and tools are predisposing factors in the prevalence of *L. monocytogenes* in Kwara state. More so, this further confirms that the bacteria can also enter to the product both during and after processing if the environment, tools and handlers are not kept hygienically. The fact that almost every household eats fried fish directly as source of protein makes it a concern in Nigeria. It is worth-noting that fried fish serves as a "bail-out" source of protein to much family whenever they have no meat in the pot. Children buy it at with little or no control. Hence, the presence of *L. monocytogenes* in the fish requires urgent attention to prevent listeriosis outbreak. Therefore, further study should be carried out to investigate the effect of cleaning and hygiene practices of the fish processors and handlers.

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