Bioresearch Communications
Volume 03, Issue 01, January 2017
Journal Homepage: www.bioresearchcommunications.com

Original Article

Isolation of muitidrug resistant *Listeria monocytogenes* from processed *Rhynchophorus phoenicis* Larvae

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ABSTRACT: *Listeria monocytogenes* was isolated from two hundred (200) samples of roasted *Rhynchophorus phoenicis* larvae samples by pour plate methods on Listeria selective agar. A total of 67 *Listeria* spp. were isolated of which 28 (41.79%) were identified biochemically and serologically as *L. monocytogenes.* All *L. monocytogenes* successfully amplified the *hly*A gene virulent markers. Antibitoics susceptibility pattern of *L. monocytogenes* showed that all isolates were resistant to cloxacillin, augumentin, ceftrizone, and amoxicillin. Other isolates showed varying antibiotics resistance pattern with 42.86% resistance to tetracycline, 21.43% for gentamycin, 35.71% for streptomycin, 50.00% for ciprofloxacin, and 46.43% for erythromycin. The study revealed that 100% of the isolates were resistant to four antibiotics while 71.43% and 57.14% of the isolates were resistant to five and more than five antibiotics.

KEYWORDS: *Listeria monocytogenes, Rhynchophorus phoenicis,* Resistant

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INTRODUCTION

Rhynchophorus phoenicis (Africa palm weevil) popularly known as edible worm is consumed either roasted or boiled by the people of southern Nigeria and other African countries where it is found. The larvae are by-product of the oil palm and palm trees from which palm wine is obtained [1]. The use of the larva of *Rhynchophorus phoenicis* is known to have both nutritional value and medicinal properties[2]. The larvae are widely hawked along major highways in Nigeria while some are sold in the markets and restaurants. Although the larvae are produced by drying, there are inherent microbiological risks associated with the display, handling, packaging and storage of the larvae prior to purchase by the final consumer. *L. monocytogenes* are very important food borne pathogens which has assumed lot of interest due to its ubiquitous nature and its ability to cause listeriosis in humans. *Listeria* spp. are wide-spread in the environment and has been isolated from soil, water, sewage, foods, humans, domestic animals, raw agricultural products and food processing environments[3]. The mode of transmission of *L. monocytogenes* is mainly by ingesting contaminated foods. Few food surveys conducted in Nigeria had reported detection of *L. monocytogenes* in both raw and ready- to-eat (RTE) foods [4, 5, 6]. Although, *L. monocytogenes* have not been reported from edible worms, other microorganisms such as *Bacillus subtilis, Staphylococcus sp., Acinetobacter sp., Pseudomonas sp. Micrococcus sp., Proteus sp., Penicillium sp., Aspergillus sp. and Saccharomyces cerevisae* have been reported from larva sold at Taabaa Ogoniland, Rivers State. However, the actual incidence of foodborne listeriosis cases in Nigeria is not known as there are no data on food poisoning or food recalls caused by *L. monocytogenes.* The objective of this study was to determine the presence of muitidrugresistant *Listeria monocytogenes* from roasted *Rhynchophorus phoenicis* larvae.

MATERIALS AND METHODS

Sampling

Sampling was carried out between February and December 2014, two hundred (200) samples of roasted *Rhynchophorus phoenicis* larvae samples were purchased from hawkers along sapele road Benin city, sapele round about, effurun round about and Ugheli- Patani road. The samples were transported to laboratory in ice packs.

Isolation and identification of *L. monocytogenes*

The ISO 11920-1[7] method for isolation and identification of *L. monocytogenes* was used with slight modifications. Ten grams of each sample was crushed in a sterile mortar then homogenized in 100ml peptone water. This was followed by pre-incubation for 4h in peptone broth after which ten (10) fold serial dilutions were then made aseptically from the homogenate then one (1) ml of aliquot was pour plated in duplicates using Listeria selective agar (Oxoid, Basingstoke, UK) and the plates were incubated at 37°C for 24-48 h. All *Listeria* species hydrolyse aesculin as evidenced by blackening of the medium hence discrete *Listeria* colonies appeared grey with black hollow zones surrounding it. The isolates were further tested for haemolysis on blood agar, carbohydrate fermentation of mannitol, rhamnose and xylose and O.B.I.S. mono (Oxoid Biochemical Identification System) (Oxoid, Basingstoke, UK).

Listeria Serotyping

Serotyping was performed using DR1126 Oxoid Polyvalent antisera (Oxoid, Basingstoke, UK) in accordance with manufacturer's instructions. DR1126 Oxoid Polyvalent antisera use were prepared against purified flagellin proteins from *Listeria monocytogenes* (antigens A, B and C) and *Listeria grayi* (antigen E) and are used to coat latex particles. When the polyvalent antisera was mixed with a suspension containing suspected *Listeria sp*, the latex particles rapidly agglutinate forming visible clumps. Absence of agglutination was indicative that the organism is not *Listeria*.

Identification of *Listeria monocytogenes*

To identify *L. monocytogenes*, genomic DNA was extracted using ZYMO® DNA extraction kit (Inqaba biotech, South Africa). PCR was performed with F-CGGAGGTTCCGCAAAAGATG and R-CCTCCAGAGTGATCGATGTT primers (Inqaba Biotech, South Africa), the primers allowed amplification of 234 bp fragment of the *hly*A gene. The reaction mixture was prepared in 0.2 ml PCR tubes with 25 µl reaction volumes and done under the following thermocycling conditions in a GeneAmp PCR system (Geneamp, Singapore) 94 °C for 4 minute for initial denaturation then 30 cycles, each at 94 °C for 30 seconds, 52 °C for 1 mintue, and 68 °C for 1 minute 30 seconds and final extension at 68 °C for 7 minutes

Antibiotic susceptibility assay resistance analysis

The antibiotic susceptibility of the isolates were determined by the disk diffusion method on Mueller-Hinton Agar (Oxoid Basingstoke, UK). The antibiotics used were ceftazidine (30µg CAZ), cefuroxime (30µg CRX), gentamycin (10µg GEN), cloxacillin (10µg CXC), augumentin (30µg AUG), ciprofloxacin(10µg CPX), ceftrizone (30µg CRO), amoxicillin (25µg AMX), streptomycin (30µg STR), tetracycline (30 µg TET) and erythromycin (30µg ERY). Isolated colonies of *L. monocytogenes* were streaked on Muller Hinton Agar after which the impregnated disk were aseptically placed on the inoculated plates and incubated at 37°C for 24 hours. After incubation the diameter (in mm) of the zone around each disk was measured and recoded (Table 2).

Detection of erythromycin and tetracycline resistant genes

Genes coding for erythromycin resistance (*eryB*), and tetracycline resistance (*tetM and tetA*) was carried out using PCR. PCR was performed with the following primers *tetM* F-GTRAYGAACTTTACCGAATC, *tetM* R-ATCGYAGAAGCGGRTCAC,
*tet*A F- TTGGCATTCTGCATTCACTC, tetA R-GTATAGCTTGCCGGAAGTCG,
ermB F – GAAAAGGTACTCAACCAAATA, ermB R- AGTAACGGTACTTAAATTGTTTAC
Each reaction mixture was prepared in 0.2 ml PCR tubes. The thermocycling conditions was 94 °C for 4 minues for initial denauration then 31 cycles, each at 94 °C for 45 seconds, 55 °C for 1 mintue, 68 °C for 1 minute and final extension at 68 °C for 8 minutes after which PCR products were separated in 1.5% agarose gel and visualized under UV light

RESULTS AND DISCUSSION

A total of 67 *Listeria* spp. were isolated from two hundred samples of *Rhynchophorus phoenicis* of

which 28 (41.79%) were identified as *L. monocytogenes* (Table1). The highest prevalence of Listeria positive samples was 22 (32.84%) of which 9 (32.14%) represented *L. monocytogenes.* *L. monocytogenes* was recovered from every sampling point in this study implying that contamination may arise after processing and these larvae could be a potential vehicle for the transmission of food borne listeriosis. The prevalence of *L. monocytogenes* in *Rhynchophorus phoenicis* larvae can be attributed to handling, processing and storage. Also the proteineous nature of the larvae can support the growth of microorganism. Although no *L. monocytogenes* have been reported from *Rhynchophorus phoenicis* larvae in previous work, other bacteria of public health importance have been isolated. Ikenebomeh and Elohor[8], reported
the isolation of *Staphylococcus auerus*, *Bacillus cereus* and *E. aerogenes* from fresh and roasted edible *Rhynchophorus phoenics* larvae from 5 locations in Delta and Edo states of Nigeria. Also, Ekrakene and Igeleke [9], isolated *Staphylococcus aureus* (100%), *Bacillus cereus* (30%), *Escherichia coli* (20%), *Enterococcus faecalis* (45%) and *Pseudomononas aeruginosa* (35%) from roasted larva of the palm weevil.
All the isolates biochemically identified as *L. monocytogenes*, successfully amplified the *hly*A gene. LLO (listeriolysin) encoded by *hly*A gene is the primary virulence factor associated with *L. monocytogenes* infection by allowing escape from the primary phagosome[10]. The presence of *hly*A gene in L. monocytogenes in this study These findings corresponds with the published work for detection of LLO in *L. monocytogenes*[11.]

Table 1. Incidence of *Listeria sp.* and *L. monocytogenes* in

Sampling point	*Listeria spp*. n (%)	*Listeria monocytogenes* n (%)
1	15 (22.39)	6 (21.42)
2	8 (11.94)	5 (17.85)
3	22 (32.84)	9 (32.14)
4	17 (25.37)	7 (25.00)
5	5 (7.46)	1 (3.57)
Total	67	28 (41.79)

The antibiotic pattern of the *Listeria monocytogenes* is shown in Table 2. All isolates were resistant to cloxacillin, augumentin, ceftrizone, and amoxicillin. Other isolates showed varying antibiotics resistance pattern with 42.86% resistance to tetracycline, 64.29% for cefuroxime, 35.71% for streptomycin, 71.43% for ceftazidime, and 46.43% for erythromycin. The resistance shown by *L. monocytogenes* strains in this study to common antibiotics of choice may be due to abuse of drugs use in animal husbandry or mutation and appearance of new strains. These results are consistent with reports of emergence of resistance and resistant genes in *Listeria sp*. Similar antibiotic pattern of *L. monocytogenes* to ciprofloxacin and gentamicin seen in this study has also been reported by Yakubu et al. [12] and Rahimi et al.[13] where over 60.0% of isolates from various sources were found to be susceptible to each of these antimicrobial agents. However, there are increasing reports of Listeria strains isolated from various sources been resistant to penicillin, ampicillin, tetracycline, streptomycin, clindamycin, oxacillin and vancomycin[14]. Yakubu et al.[12] reported 100% resistance to ampicillin from isolates in milk and processed meat. Although all strains of *L. monocytogenes* in this study were resistant to augumentin. Umoh et al.[15] reported all strains as augumentin susceptible suggesting that genetic differences may occur within the strains of *L. monocytogenes* while Heger et al.[16] and Troxler et al.[17], reported that newer generations of cephalosporins, like ceftriaxone, have no in-vitro effect on *L. monocytogenes* membranes. Resistance to tetracycline and erythromycin observed in this study has also been reported in *L. monocytogenes* isolates from foods [18, 19].

Table 2. Antibiotics pattern of *L. monocytogenes* from *Rhynchophorus phoenicis*

Antibiotics	Susceptibile n (%)	Resistant n(%)
Ceftazidine (CAZ)	8 (28.57)	20 (71.43)
Cefuroxime (CRX)	10 (35.71)	18 (64.29)
Gentamycin (GEN)	22 (78.57)	6 (21.43)
Cloxacillin (CXC)	0 (0.00)	28(100.00)
Augumentin (AUG)	0 (0.00)	28(100.00)
Ciprofloxacin (CPX)	14 (50.00)	14 (50.00)
Ceftrizone (CRO)	0 (0.00)	28(100.00)

Amoxicillin (AMX)	0 (0.00)	28(100.00)
Streptomycin (STR)	18 (64.29)	10(35.71)
Tetracycline (TET)	16 (57.14)	12 (42.86)
Erythromycin (ERY)	15 (53.57)	13 (46.43)

The multiple drug resistance of the *L. monocytogenes* is shown in Table 3 with 100% of the isolates been resistant to four antibiotics. This resistance shown by *L. monocytogenes* strains in this study may be due to abuse of drugs, use in animal husbandry, mutation and appearance of new strains Resistance to five and more than five antibiotics represented 71.43% and 57.14% respectively. This result is in agreement with the findings by other researchers who reported multidrug resistance among *Listeria monocytogenes*[12, 20]. Multiple antimicrobial resistance in *Listeria monocytogenes* is reported to be mediated by self transferable plasmids[21].

Table 3. Multiple drug resistance of the *L. monocytogenes*

Antibiotics	frequency	percent
Resistance to four antibiotics	28	100.00
Resistance to five antibiotics	20	71.43
Resistance to more than five antibiotics	16	57.14

The occurrence of antimicrobial resistance genes in *L. monocytogenes* is shown in figure 1. 30% of the isolates carried ermB genes while 66.66% and 58.92% of the isolates carried *tetA* and *tetM* respectively. tetM is known to confer resistance by ribosomal protection, while *tetA* confer resistance by efflux[21]. Similar report has shown that tetM and *tetA* genes were present in 91.7 and 83.3 % of the tetracycline resistant *Listeria* [22]. The prevalence rate of the *tetM* and *tetA*, in the present study is also in agreement with the investigations in which a high frequency of these resistance genes in *L. monocytogenes* isolates was reported [21,23,24,25]. In *Listeria spp.*, two genes for resistance to macrolide-lincosamide-streptogramin B (MLS_B), *erm*(B) and *erm*(C) have been reported[21]. These *erm* (*e*rythromycin *r*ibosome *m*ethylase) genes encode methyltransferases that modify 23S rRNA in bacteria. Erythromycin resistant genes detected in this study are in accordance to reports by Roberts et al.[26] who reported the presence of *erm(c)* genes in *L. monocytogenes*.

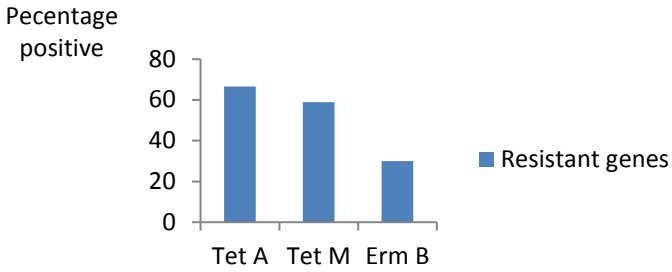

Figure 1. Occurrence of antimicrobial resistance genes *in L. monocytogenes*

In conclusion, the results obtained from this study showed that *Rhynchophorus phoenicis* larvae available to consumers may be contaminated with multi resistant *Listeria monocytogenes* awhich may lead to human infection as reported in several countries. Hence, unhygienic practices during processing and sales should be discouraged.

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