

Original Research Article

Phenotypic and Genotypic Virulence Potentials of *Listeria Monocytogenes* Isolated from Different Food Samples in Yola

ABSTRACT

Background: Foodborne listeriosis caused by *L. monocytogenes* is one of the most serious and severe foodborne diseases. The severity of *L. monocytogenes* infection is associated with several factors which include the expression of virulence factors. Determination of the pathogenic potential of *L. monocytogenes* is important from food safety and public health perspective. **Aim:** the aim of this work is to determine the Phenotypic and Genotypic Virulence Potentials of seven *Listeria Monocytogenes* Isolated from Different Food Samples in Yola. **Methodology:** phenotypic virulence potentials of the isolates was determined by haemolytic activity on 5% sheep blood agar and biofilm formation ability, using Christensen's tube method and microtiter plate method for the qualitative and quantitative biofilm formation respectively. Genotypic virulence determination was done by polymerase chain reaction amplification of *plcB*, *hlyA* and *actA* genes. **Result:** All the isolates (100%) were beta haemolytic on 5% sheep blood agar and all the isolates were biofilm formers but with different degree of biofilm formation abilities. Genotypically all the isolates (100%) indicated the presence of all the three virulence genes amplified. **Conclusion:** Food samples analyzed isolates are potentially virulence pathogenic of *L. monocytogenes* that can pose food borne listeriosis to high risk individuals in the study area.

Keywords: Foodborne genes genotypic listeriosis *L. monocytogenes* pathogenic phenotypic virulence

1. INTRODUCTION

Listeria monocytogenes is an important, ubiquitous, foodborne bacterium that can contaminate food products during or after processing. It poses a significant risk to the food industry, particularly producers of ready-to-eat (RTE) foods (of vegetables and animal origin) due to its ability to proliferate over a vast range of adverse environmental conditions [1]. Hence, it has become a major concern for the food industry.

The scientific interest in *Listeria* derives from the fact that the virulent species are facultative intracellular pathogens, enabling them to survive within host cells, similar to pathogenic *Mycobacteria*, *Salmonella*, *Shigella*, *Legionella*, and several other important intracellular bacterial pathogens [2]. Virulence factors in the infectious cycle of pathogenic *Listeria* are encoded on a 9-kb pathogenicity island known as *PrfA*-dependent virulence gene cluster or LIPI-1 (*Listeria* pathogenicity island 1) [3]. This chromosomal locus comprises the genes : (i)

hly, encoding a cholesterol-binding, pore-forming toxin (listeriolysin O, LLO) essential for the bacterial escape from phagosome; (ii) *plcA* and *plcB*, encoding two phospholipases C with different substrate specificities (*PlcA* is phosphatidyl inositol specific and *PlcB* is a lecithinase with a broad substrate range) and which co-operate with LLO in the escape from phagocytic vacuoles; (iii) *mpl*, encoding a metalloprotease involved in the maturation of pro-*PlcB*; (iv) *actA*, coding for the ActA protein which mediates actin-based intra- and intercellular motility of the bacteria; and (v) *prfA*, which encodes PrfA (positive regulatory factor A). This protein activates transcription of all genes of *LIPI-1*, including its own [4].

In the cytoplasm, *L. monocytogenes* replicates rapidly and produces the surface actin assembly-inducing (ActA) protein [5]. ActA induce actin formation creating a comet tail, ultimately pushing the bacterium towards the host cell surface to invade neighboring cells. In this manner, *L. monocytogenes* replicates and spread within the host avoiding the extracellular space and evading the immune system[4].

Pathogenic *Listeria* spp. produces three different enzymes with phospholipase C (PLC) activity that is involved in virulence. Two, *PlcA* and *PlcB*, are present in *L. monocytogenes* and *L. ivanovii*; the third, SmcL, is specific to *L. ivanovii*. Key virulence factor essential for pathogenicity, having a vital role not only in intracellular parasitism but also in several other functions in the interaction of *Listeria* with their vertebrate host[4].

Haemolysin of *L. monocytogenes* is an SLO-related cytolysin belonging to the family of cholesterol-dependent, pore-forming toxins (CDTX). This toxin was given the name listeriolysin O (LLO), and one of its key characteristics was determined, its low optimum pH (5.5) and the narrow pH range at which it is active (4.5 to 6.5). LLO not only mediates lysis of the primary phagosomes formed after the uptake of extracellular bacteria but is also required for the efficient escape of *L. monocytogenes* from the double-membrane vacuole that forms upon cell-to-cell spread. The pores or membrane lesions caused by LLO probably facilitate the access of *Listeria* phospholipases to their substrates, leading to total dissolution of the physical barrier that delimits the phagosomal compartment [4].

2. MATERIAL AND METHODS

2.1 Source of organisms

The organisms *Listeria monocytogenes* were previously isolated from different food samples obtained from Yola, the Capital of Adamawa state.

2.2 Determination of Phenotypic Virulence Potentials of *L. monocytogenes*

2.2.1 Qualitative Determination of Biofilm Formation

Biofilm formation was determined using Christensen's tube method, where 1ml of the standardized inoculum was introduced into test tubes containing 10ml of nutrient broth and incubated for 24 hours at 37 °C without shaking. After 24 hours of incubation, the tubes were decanted and washed with PBS (pH 7.3) and dried. The dried tubes were stained with 0.1 % crystal violet, the excess stain was removed, and the tubes were washed again with deionized water. The tubes were dried in an inverted position and observed for slime layer formation. Biofilm formation was considered positive when a visible film lined the wall and bottom of the tube. Ring formation at the liquid interface was not considered biofilm formation. All the isolates were tested in triplicate. Tubes were examined for crystal violet retention. The amount of biofilm formation was recorded as 0-absent, 1-weak, 2-moderate, or 3-strong based on the intensity of crystal violet retention in the tubes [6].

2.2.2 Quantitative Determination of Biofilm Formation

The microtiter plate method was used for the quantitative measurement of biofilm. Overnight grown colonies were diluted to obtain an OD₆₀₀ of 0.1 in each growth medium and 200 µl of bacterial solution was transferred in triplicate wells in 96-well microplates. Microplates were incubated without shaking for 24 h at 37°C. Plates were inverted and the media and planktonic cells were removed by gentle tapping. To remove loosely attached bacteria, wells were washed twice with 300 ml of sterile saline solution (8.5 g NaCl per liter). Then biofilms were fixed with 300 µl of 96% v/v ethanol for 20 min and air-dried completely at room temperature after removal of the ethanol. For staining bacterial biomass, 220 µl of 0.1% w/v crystal violet (CV) solution was added per well, and plates were incubated also without shaking for 30 min. Then the solution was removed by sharply tapping the plates upside down. Wells were washed 3 times with 300 µl of saline and air dried completely before filling with 150 µl of 33% v/v acetic acid. Plates were placed on a plate shaker with slight agitation for 10 min to completely dissolve CV and get homogenized solutions. The amount of destained CV was determined by reading OD₆₀₀ in a microplate reader [7]. Optical Density (O.D)₆₀₀ < (0.1AU = Weak); (0.1-0.4AU = moderate); (>0.4- 1AU= Strong); (> 1AU = Very Strong).

2.2.3 Haemolysis test

Standardized inoculums of *L. monocytogenes* isolates were streaked on 5% sheep blood agar and incubated at 37°C for 24 hours after which zone of β-haemolysis on the blood agar was observed [8].

2.3 Determination of Genotypic Virulence potentials of *L. monocytogenes*

2.3.1 DNA extraction

Freshly grown *L. monocytogenes* colonies collected from the surfaces of 24-hour nutrient agar culture plates were used for DNA extraction [9]. Qiagen QIAamp DNA mini kit was used and the DNA extraction procedure was according to the manufacturer's instructions.

2.3.2 PCR Amplification and Detection of Virulence Genes

The isolates were screened by multiplex PCR for the presence of some of the virulence genes (*hlyA*, *plcB*, and *actA*) using the primers: *hlyA* F: 5'-GCA GTT GCA AGC GCT TGG AGT GAA-3', R: 5'- GCA ACG TAT CCT CCA GAG TGA TCG-3' [10] to amplify the 456-bp fragment; *plcB* F: 5'-CTG CTTGAG CGT TCA TGT CTC ATC CCC C-3', R: 5'-ATG GGT TTC ACT CTC CTT CTA C-3' [11] to amplify the 1484-bp fragment and *actA* F: 5'-CGC CGC GGA AATTAA AAA AAG A-3', R: 5'- ACG AAG GAA CCG GGC TGC TAG - 3' [12] to amplify the 839-bp fragment. Amplification was performed in 50 µL reaction volume as described by [11] containing 10 µL (30–50 ng) of extracted DNA template from the identified *L. monocytogenes* cultures, 5 µL 10×PCR buffer, 5 µL MgCl₂ (25 mM), 4 µL dNTPs (25 mM), 1.54 µL (2.5 U/ µL) Ampli Taq DNA polymerase, 2 µL (0.5 µM) from each primer pairs. The volume of the reaction mixture was completed to 50 µL using Double Distilled Water (DDW) and the tubes were placed in a DNA thermal cycler (SelectCycler II). The samples were subjected to an initial denaturation step of 95°C for 2 min, followed by 35 amplification cycles of 15s at 95°C (denaturation), 30s at 60°C (annealing), and 90s at 72°C (primer extension) followed by a final extension step of 72°C for 10 min.

2.3.3 Separation of the PCR Product

Electrophoresis' separation of the PCR product was performed on 1.5 % agarose gel, which was stained with ethidium bromide. The sizes of the PCR products were also compared with a standard 100bp DNA marker. A reaction mixture with no DNA template was incorporated as a negative control. The process was performed at 110 V and 1.81 A for 1 hour and 15 minutes, and the gel was observed under ultraviolet light [11].

3. RESULTS AND DISCUSSION

3.1 Phenotypic Virulence Potentials of *Listeria monocytogenes* Isolates

3.1.1 Biofilm Formed by *L. monocytogenes* isolates

Both Christensen's tube method and microtiter plate methods for the determination of biofilm formation ability revealed that all the isolates are biofilm formers but with different degree of biofilm formation abilities as shown in Table 1.

Table 1. Biofilm Formation Ability of *L. monocytogenes* isolates

s/n	Isolates Code	Degree of Biofilm Formation	
		Microtiter Plate Assay(OD ₆₀₀)	Christensen's tube Method
1	Lm 2C	Moderate	Moderate
2	Lm 3C	Strong	Strong
3	Lm 4C	Moderate	Moderate
4	Lm 8Fc	Moderate	Moderate
5	Lm 9Fc	Moderate	Strong
6	Lm 10Fc	Moderate	Moderate
7	Lm 11Y	Strong	Moderate

*Microtiter Plate Assay; Optical Density (OD₆₀₀) < 0.1AU = Weak); (0.1-0.4AU = moderate); (>0.4-1AU = Strong); (> 1AU = Very Strong). A.U = Absorbance Unit. Christensen's tube Method; base on the intensity of crystal violet retention in the tubes, 0-absent, 1-weak, 2-moderate or 3-strong

The *L. monocytogenes* isolates in this work showed different degrees of biofilm formation ability at room temperature (25-30°C); from weak, to moderate and strong. Most of the isolates (45.5%) showed moderate biofilm formation ability, some (18.1%) showed strong ability, while others (36.3%) had weak biofilm formation ability. However, there are discrepancies in the qualitative and quantitative values of biofilm observed in some isolates. This may be because the two methods, measured biofilm formed on different media (glass and polystyrene) and the organism have different adhering ability to the different media. They adhere preferentially, to glass which is less hydrophobic than polystyrene. More so, the qualitative method (Christensen's tube method) is prone to human error as no machine is involved in reading the result. Therefore, the quantitative method (microtiter plate method) was used to identify the biofilm formers in this study. Similarly, [13] analyzed biofilm formation of *L. monocytogenes* on different surfaces at different temperatures and observed complex organization of *L. monocytogenes* biofilms at 22 and 37 °C in terms of cell number and extracellular polymeric substance (EPSs) produced. Whereas, a rudimentary biofilm consisting of a sparse cluster of cells and few EPSs were observed at 4 and 12 °C with higher levels on glass compared to the more hydrophobic stainless steel and polystyrene. [14] also reported that *L. monocytogenes* significantly formed biofilm at 25, 37°C.

This finding indicates that *L. monocytogenes* can form biofilm on several surfaces used in the food industry at different temperature, representing a serious alarm for food safety because it could serve as a source of contamination. *L. monocytogenes* can attach to many food-contact surfaces, such as stainless steel, polystyrene and glass [13]. It has been found to persist in food industries for several years, where it could cause recurrent cross-contamination of food products [15]. When organized as biofilm, the self-produced extracellular polymeric matrix gives extra protection to bacteria from harsh environmental conditions such as desiccation, nutrient deprivation, or disinfectant treatment [16]. As a consequence, it is challenging to control bacterial contaminations in the Food Processing Environment (FPE) [7].

3.1.2 Haemolytic Characteristics of *L. monocytogenes* Isolates

Zone of β -haemolysis on 5% sheep blood agar was produced by all the isolates identified as *L. monocytogenes* indicating that all the isolates are haemolytic.

Haemolysis is one of the determining factors of virulence and pathogenicity of many haemolytic bacteria. All isolates in this study showed β -haemolytic activity; this indicates that they are pathogenic. This is not surprising because, previously, food isolates were tested with a tissue culture model and shown to be cytopathogenic indicating their pathogenicity [17]. Based on that, the absence or presence of this virulence factor could be a tool to assess the risks related to food product consumption [18].

3.2 Genotypic Virulence of *L. monocytogenes* Isolates

3.2.1 Virulence Genes Detected from *L. monocytogenes* Isolates

The molecular detection of virulence genes *hlyA*, *plcB*, and *actA* from the *L. Monocytogenes* isolates with more phenotypic virulence potentials (biofilm formation ability and haemolysis), indicated the presence of all the three virulence genes in all the isolates. Fig. 1 (a) and (b) present gel pictures of the amplified virulence genes in all the isolates.

Previous studies conducted on several types of food isolates have documented similar results of incidence of virulence genes in all examined *L. monocytogenes* isolates [19][20][21]and [22]. Also [23] reported variable rates of virulence gene detection in *L. monocytogenes* isolates. In addition, other studies also confirmed similar findings in samples isolated from diverse types of food, raw milk, milking machines, workers' hands and clinical specimens [24][25][26].

Although, some polymorphisms and punctual mutations may be present in certain virulence genes and their presence may contribute in favour of attenuated virulence from *L. monocytogenes* strains [27]. The presence of the virulence genes *hlyA* in all of the isolates, justified the phenotypic haemolytic activity observed in all the isolates. Therefore, the presence of these virulence genes (*hlyA*, *ActA* and *plcB*) and other virulence factors such as biofilm formation and haemolysis in all the isolates, demonstrate the potential pathogenicity of these isolates.

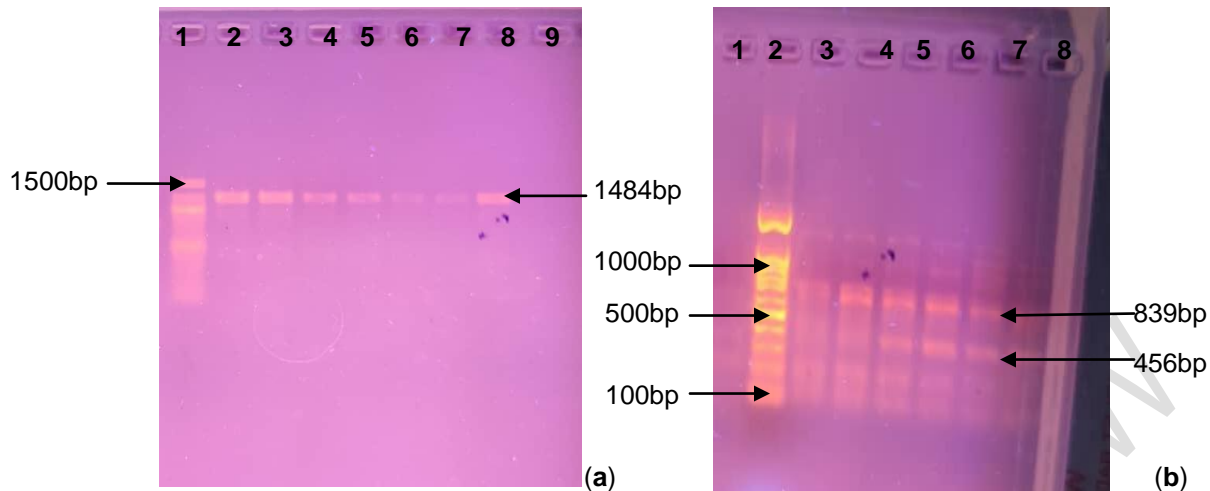


Fig. 1(a); Gel picture of an amplified *plcB* gene (1484 bp). Lane 1: Molecular weight Marker (100 bp); Lanes 2-8: positive isolates and Lane 9: Negative control **Fig. 1(b);** Multiplex PCR products of an amplified *hlyA* gene (456 bp) and *actA* gene (839 bp). Lane 3-8 positive isolates for *actA* and *hlyA*; Lane 2: Molecular weight Marker (100 bp); Lane 1: Negative control.

4. CONCLUSION

The food samples isolates are potentially virulence pathogenic of *L. monocytogenes* that can pose food borne listeriosis to high risk individuals in the study area. The finding provides evidence of a serious public health issue since this organism represents a potential threat to consumers of all the *L. monocytogenes*-positive food samples studied, especially those from the risk groups (pregnant women, elderly and immunocompromised individuals).

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