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

ABSTRACT

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. **Study design:**the study was a randomized study; the samples (sources of organisms) were collected at random from different market and shops within the study area. **Place and Duration of Study**:the study was carried out in Yola, the capital of Adamawa State, north eastern Nigeria. **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 isolates analyzed 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.

"Listeria species has been associated with a wide variety of food sources particularly poultry, red meat, and meat products. In Egypt, *Listeria* contamination rate in meat and chicken products was reported to be 41%, lower than the 73.9% reported in Malaysia from imported frozen beef and 83.3% from raw minced meat in Turkey" [2, 3]. "The bacteria can be endemic in food processing environments, because it survives food-processing technologies that rely on acidic or salty conditions and, unlike many pathogens, can continue to multiply slowly at low temperatures, allowing for growth even in properly refrigerated foods. Hence, their presence may be indicative of poor hygiene or cross contamination, which is considered a possible source of *Listeria* contamination in processed meat"[4].

"L. monocytogenes is the main cause of food borne listeriosis in humans. Rarely, food borne infections were reported by *L. ivanovii* and *L. seeligeri*. Strains of *L. monocytogenes* have different pathogenic potential, as some strains are very virulent, whereas some of them are non-infectious agents"[5]. "Determination of the pathogenic potential of *L. monocytogenes* is important from food safety and public health perspective"[6]. "Listeriosis has emerged as an atypical food borne illness of major public health concern because of the severity of the disease (meningitis, septicemia, and abortion), the high case fatality rate (20–30% of cases), the long incubation period, and the predilection for individuals who have an underlying condition which leads to impairment of T-cell-mediated immunity" [7, 8].

"Listeria monocytogenes has been recovered from several foods such as meat, milk, and fish products; ice cream; vegetables; and several ready-to-eat foods" [9][10]. "*Listeria monocytogenes* is responsible for cases and outbreaks of febrile gastrointestinal disease in otherwise healthy people and invasive listeriosis, which usually affects pregnant women, newborns, the elderly and immunocompromised individuals. The genomic epidemiology of *L. monocytogenes* has been investigated and genetic differentiation of the organism within and between food categories was calculated based on allele frequencies in each food category. It has been found that some clonal complexes (CCs) are overrepresented but could not identify any epidemiological risk factors. This suggested persistent contamination in food production settings, and producers that process a wide variety of raw food produce, could significantly contribute to lowering the *L. monocytogenes* disease burden"[11].

"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" [12]. "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)"[13]. "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"[14].

"In the cytoplasm, *L. monocytogenes* replicates rapidly and produces the surface actin assembly-inducing (ActA) protein"[15]. "*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"[14].

"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"[14].

"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)". [43]"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"[14].

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.The samples are cabbage, frozen chicken, fresh fish, raw meat, ice-cream, fruit salad and yoghurt.

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 [15].6

2.2.2Quantitative Determination of Biofilm Formation

The microtiter plate method was used for the quantitative measurement of biofilm. "Overnight grown colonies were diluted to obtain an OD_{600} 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 37^0C. 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 OD_{600} in a microplate reader"[16]7].Optical Density (O.D) $_{600}$< (0.1AU = Weak); (0.1-0.4AU = moderate); (>0.4-1AU= Strong); (> 1AU = Very Strong).

2.2.3Haemolysis test

"Standardized inoculums of *L. monocytogenes* isolates were streaked on 5% sheep blood agar and incubated at 37^0C for 24 hours after which zone of β-haemolysis on the blood agar was observed" [17]8.

2.3Determination 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 [18] 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 in Table 1 below.

Table 1: Primers used for PCR Amplifications of Virulence Genes

Gene	Primers	Gene size (bp)	Reference
*hly*A	(for.) GCA GTT GCA AGC GCT TGG AGT GAA (rev.) GCA ACG TAT CCT CCA GAG TGA TCG	456	[19] 10]
plcB	(for.) CTG CTT GAG CGT TCA TGT CTC ATC CCC C (rev.) ATG GGT TTC ACT CTC CTT CTA C	1484	[20]11
*act*A	(for.) CGC CGC GGA AAT TAA AAA AAG A (rev.) ACG AAG GAA CCG GGC TGC TAG	839	[21]12

Amplification was performed in 50μL reaction volume as described by [20] containing 10 μL (30–50 ng) of extracted DNA template from the identified *L. monocytogenes* cultures, 5 μL 10×PCR buffer, 5 μL $MgCl_2$ (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 [20] 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 2.

Table 2. 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 (OD_{600})< (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 (25-300C); 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, [22] 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. [23]14] also reported that *L. monocytogenes* significantly formed biofilm at 25, 370C.

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"[22]. "It has been found to persist in food industries for several years, where it could cause recurrent cross-contamination of food products"[24]. "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"[25]. "As a consequence, it is challenging to control bacterial contaminations in the Food Processing Environment (FPE)"[18]."The ability of foodborne pathogens, including *L. monocytogenes*, Yersinia enterocolitica, Campylobacter jejuni, and Escherichia coli O157:H7 to attach to food and food-contact surfaces is well known. Some authors have found that *L. monocytogenes* has become one of the major causes of food product contamination and disease transmission"[26][27]. "In 2011, there was an outbreak linked to whole cantaloupe contaminated with *L. monocytogenes*. It was speculated that the root cause of the outbreak was the unsanitary condition of the packing shed, but the microorganisms were also found in other places, including on the conveyor belt, and in the drying area and the floor drain. In 2018, a rock melon *L. monocytogenes* outbreak in Australia killed four people"[28][29]. Latorre et al. [30] conducted "a study on the presence of *L. monocytogenes*–containing biofilm in milking equipment as a potential source of contamination on a dairy farm". "After obtaining positive results, electron microscopy scanning of the equipment showed the presence of individual cells and clusters of bacteria and it was postulated that they were mainly associated with scratched surfaces"[29].

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 [32]. Based on that, the absence or presence of this virulence factor could be a tool to assess the risks related to food product consumption [33].

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 [34][35][36]and [37]. Also [38] 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 [39][40][41].

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 [42]. 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, demonstrated the potential pathogenicity of these isolates.

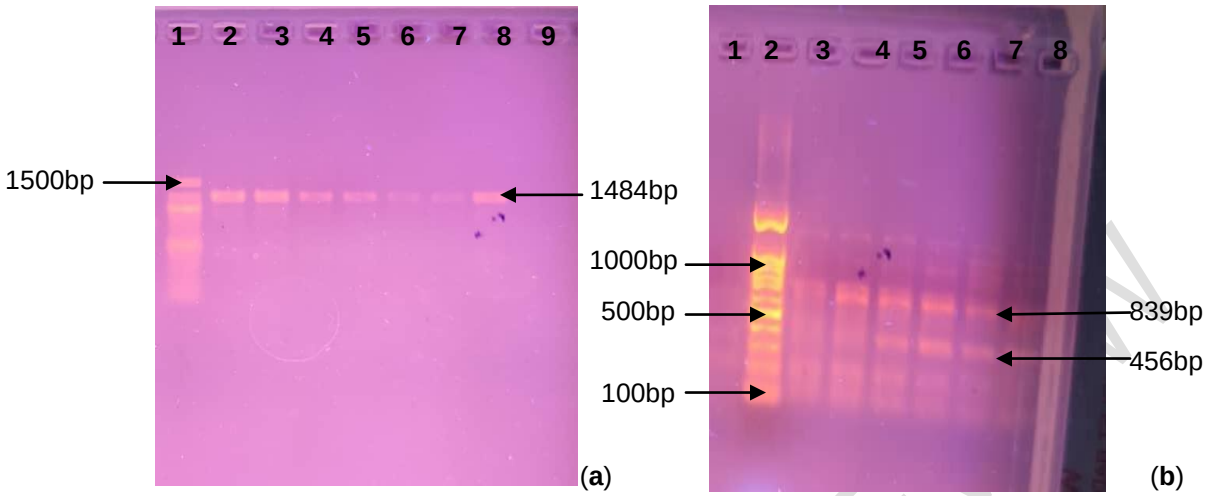

Fig. 1(a); Gel picture of an amplified plcB gene (1484 bp). Lane 1: Molecular weight Marker (100 bp); Lanes 2-8: positive isolates and Lane 9: Negative control **Fig. 1(b);** Multiplex PCR products of an amplified *hlyA* gene (456 bp) and *actA* gene (839 bp). Lane 3-8 positive isolates for *actA* and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

4. CONCLUSION

He 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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