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Research Article

PHYLOGENETIC CHARATERIZATION OF *LISTERIA MONOCYTOGENES* ISOLATED FROM DIFFERENT SOURCES IN IRAQ

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ABSTRACT

Objective: The main goal of the current study was to isolate and detect the phylogenetic characterization of *Listeria monocytogenes* isolate from clinical and non-clinical specimens.

Methods: *L. monocytogenes* was isolated from 353 samples including: (94) Vaginal swabs, (81) piece of placenta, (51) frozen chicken, (69) soft cheese, and (58) frozen red meat samples using the Association of Official Agricultural Chemists method. Polymerase chain reaction (PCR) was performed for the detection of virulence gene *hlyA* followed by DNA sequence analysis for this gene.

Results: A total of 13 isolates of *L. monocytogenes* were isolated from 2 (2.1%) vaginal swabs, 4 (4.9%) piece placenta, 2 (3.9%) frozen chicken, 3 (4.3%) frozen soft cheese, and 2 (3.4%) frozen red meat samples, and *hlyA* gene was detected in 100% of *L. monocytogenes* isolates.

Conclusion: All the isolates tested positive for *hlyA* gene, so this is important role in the study of the *L. monocytogenes* infection and its pathogenicity. The phylogenetic analysis showed a clear convergence in the *L. monocytogenes* isolates from chicken, red meat, and soft cheese 70%, while there is a marked difference in isolates of *L. monocytogenes* isolated from placenta and vaginal swabs.

Keywords: *Listeria monocytogenes,* Phylogenetic, Polymerase chain reaction, *hlyA* gene sequence.

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INTRODUCTION

Bacterial nosocomial infections can pose a main risk in immune-compromised patients. Advancement in medical sciences has led to the increased use of invasive devices resulting in high rates of infection [1]. *Listeria monocytogenes* is a rod-shaped food-borne pathogen that is gram-positive, non-spore-forming, and strictly anaerobic bacteria. This opportunistic intracellular bacterium causes one of the most common and serious worldwide food infections called Listeriosis [2,3].

Listeriosis is one of the most major bacterial diseases, related with high mortality rates of 20–30% globally. The CDC reported that it is the third leading death causing disease due to mainly contaminated food [4,5]. Listeriosis can be either non-invasive, that happened in healthy individuals causing self-cure febrile gastroenteritis, or it can be invasive where it can affect immunocompromised persons such as the older men, pregnant women, and newborns, resulting in various health conditions including encephalitis, meningitis, preterm birth, miscarriage or stillbirth in pregnant women, septicemia in neonates, and neurotic system infections, and in severe cases, it may lead to high mortality [6,7]. Conceive women are more susceptible to the *L. monocytogenes* infection because of their low immunity defense system during pregnancy. The relative risk of Listeriosis in pregnant women is 18 times more than in non-conceive women [8]. In addition, *L. monocytogenes* are more excite in nature, capable of adapting to various environmental conditions which enables it to replicate in extreme conditions including dry environments, low temperatures, a wide range of pH, and high salt concentrations as well as their ability to form biofilms, which makes it difficult to avoid food contamination [9-11]. In the processing of food, it is good to take proper measures for ensuring its safety and stability during the shelf-life [12]. Food contamination caused by *L. monocytogenes* occurs at several stages during the production and processing cycles of food. Food survey studies have demonstrated food contamination caused by *Listeria* often begins within the food processing environment during the processing or the post-processing process of food, rather than being due to survival during the processing itself [13].

METHODS

Isolation and identification of *L. monocytogenes*

From November 2016 to May 2017, 353 samples were collected from clinical specimens including vaginal swabs (n=94), placental tissue (n=81), and non-clinical specimens including frozen chicken (n=51), frozen red meat (n=58), and frozen soft cheese (n=69). The vaginal swabs and 25 g of the placental tissue as well as the non-clinical specimens were inoculated into 10 ml and 225 ml of tryptic soy broth positive 0.6% yeast extract (TSBYE) broth (HiMedia, India), respectively. After inoculation in TSBYE, all samples were homogenized and incubated for 24 h at 37°C. Subsequent to incubation, a loopful of the subsequent culture was streaked into the surface of different agar plates (PALCAM, Oxoid) *Listeria* agar, supplemented with 5 mg polymixin B, 20 mg acriflavin, and 10 mg ceftazidime and incubated at 35°C for 24-48 h according to AOAC method [14]. Bacterial identification was made using the biochemical test, oxidase test, catalase test, CAMP test, and motility test at 25°C and streaking on blood agar as described by Hitchins [15].

Detection by polymerase chain reaction (PCR) assay

The PCR was used to detect the virulence factor genes of bacteria *L. monocytogenes* isolated from clinical and food samples, according to the steps described by Borucki and Call [16].

Bacterial genomic DNA extractions

Bacterial genomic DNA of *L. monocytogenes* isolates was extracted using (genomic Mini DNA Bacteria Kit, Geneaid, USA) and done according to company instructions.

PCR

PCR was performed to detect *hlyA* gene encoding listeriolysin O (LLO) of *L. monocytogenes* using primer pairs, as shown in Table 1.

Preparation of PCR master mix

A PCR reaction mix was prepared using the AccuPower® PCR Master Mix kit, manufactured by the Korean company Bioneer, as per the company's instructions. All PCR reactions were performed in a final volume of 20 µL using 5 µL of extracted DNA as template and forward primer (10 pmol) (1.5 µL), reverse primer (10 pmol) (1.5 µL), and PCR water (12 µL). Then, PCR amplification was carried out in a programmed thermocycler with the following thermal conditions included an initial denaturation at 95°C for 3 min, followed by 31 cycles each of 30 s denaturation at 95°C, 15 s annealing at 58°C, 1 min extension at 72°C, and final extension at 72°C for 5 min, and then, the PCR product was held at 4°C. The resultant PCR products were separated by electrophoresis in 1.5% agarose gel stained with ethidium bromide and visualized with a UV transilluminator.

DNA sequencer method

The DNA sequencing method was carried out to perform the definitive diagnosis. *L. monocytogenes* was diagnosed by the PCR examination, by conducting the phylogenetic tree analysis based on *hlyA* gene. After the PCR reaction, the PCR reaction was sent to Macrogen in South Korea for a procedure sequence of DNA using the AB DNA sequencing system.

RESULTS

Isolation and identification of *L. monocytogenes*

A total of 353 samples have been collected and tested, between December 2016 and May 2017. Only 13 samples of the total have shown positive growth for *L. monocytogenes* through culturing method and confirmed by PCR as shown in Table 2.

Bacteria cells appear Gram-positive rods-like shape, singly arranged in short chains, an in pairs at V-Y-L form and in groups that were parallel to each other. On the blood agar, colony was streaked on a 5% sheep blood agar, then incubated for 24–48 h duration at 37°C. Positive results were obtained which shown by the formation of narrow zone of β-hemolysis around the colony. All samples were streaked on PALCAM, which is a selective media recommended for the isolation of *L. monocytogenes* from clinical and non-clinical samples. *Listeria* colonies appeared gray-green with a black halo against a cherry red back ground. All colonies *L. monocytogenes* were confirmed by being positive for catalase and motility at 25°C, negative for oxidase, and positive for CAMP test with *Staphylococcus aureus* as shown in Table 3.

Table 1: Primers sequences used for gene amplification

Primer	Primers sequences		Amplicon
Hly	F	AGGGGTGGCAAACGGTATTT	239 bp
	R	CATCCGCGTGTTTCTTTTCGA	

Confirmation of *L. monocytogenes* by PCR

The DNA obtained was used for the detection of *hlyA* gene in *L. monocytogenes* isolated from clinical specimens and food samples. The PCR results in the present study detected the presence of *hlyA* gene among all 13 L. *monocytogenes* isolates.

Nucleotide accession number

Five sequences PCR samples used in this study based on the *hlyA* gene sequences have been deposited in GenBank under accession numbers as shown in Table 4.

Phylogenetic tree analysis

The DNA sequence technique is used to analyze the phylogenetic tree for 5 samples of *L. monoytogenes* based on the *hlyA* gene, using the NCBI blast program, MEGA 6, and UPGMA tree analysis (unweighted pair group method with arithmetic mean) as shown in Figs. 1 and 2.

DISCUSSION

L. monocytogenes causes *Listeriosis* is characterized by septicemia and encephalitis which can consequently lead to abortion or defects during the development of babies before birth. The ability of *L. monocytogenes* to persist in food-processing environments and its ability to grow at low temperatures make this pathogen a major threat to public health, particularly in frozen foods [17].

In this study, the diagnosis of *L. monocytogenes* isolates from clinical and food samples was confirmed using PCR, identified 13 L. *monocytogenes* isolates sheltering *hly A* gene, which agree with other previous studies such as [18] in Egypt who mentioned that all *L. monocytogenes* isolates are positive for *hlyA* gene from meat. Furthermore, Kamar *et al*. [19], in Egypt, detected *hlyA* gene in all *L. monocytogenes* isolated from different sources. Moreover, Lotfollahi *et al*. [20], in Iran, found the prevalence of *hlyA* is 100% from dairy and meat products. However, Kargar and Ghasemi [21] reported 91.7% of the *hlyA* gene present in cheese samples. The gene (*hlyA*) is the first virulence gene identified in *Listeria* which encodes LLO. LLO is the main bacterial determinant responsible for the escape of *L. monocytogenes* from primary and

Table 2: Prevalence of *L. monocytogenes* in different source

Sample	Total n (%)	Positive samples n (%)	Negative samples n (%)	p value
Placenta tissue	81 (23)	4 (4.9)	77 (95.1)	<0.0001[S]
Vaginal swab	94 (27)	2 (2.1)	92 (97.9)	<0.0001[S]
Frozen Soft cheese	69 (20)	3 (4.3)	66 (95.7)	<0.0001[S]
Frozen red meat	58 (16)	2 (3.4)	56 (96.6)	<0.0001[S]
Frozen chicken	51 (14)	2 (3.9)	49 (96.1)	<0.0001[S]
Total	353	13 (3.7)	340 (96.3)	

S: Significant association, *L. monocytogenes: Listeria monocytogenes*

Table 3: Biochemical tests of *L. monocytogenes*

Sample	Positive samples	Catalase	Oxidase	Motility at 25°C	CAMP test
Placenta tissue	4	+	_	+	+ S
Vaginal swab	2	+	_	+	+ S
Frozen soft cheese	3	+	_	+	+ S
Frozen red meat	2	+	_	+	+ S
Frozen chicken	2	+	_	+	+ S
Total	13				

p<0.0001, *L. monocytogenes: Listeria monocytogenes*

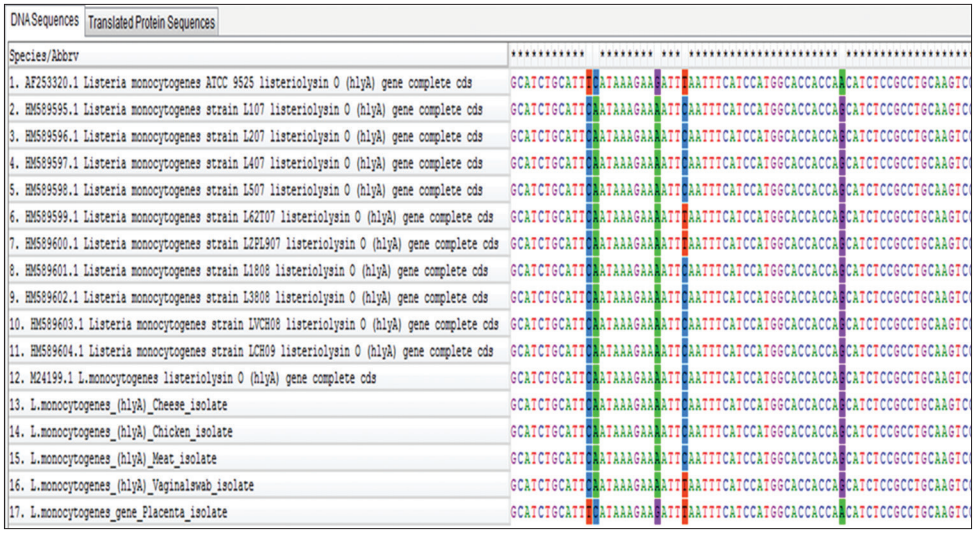

Fig. 1: Multiple sequence alignment of *Listeria monocytogenes* (the present study) isolated from 1 to 5 with the closely related isolates from different sources. Only variable sites are shown with different color. Dashes in the middle indicate gaps

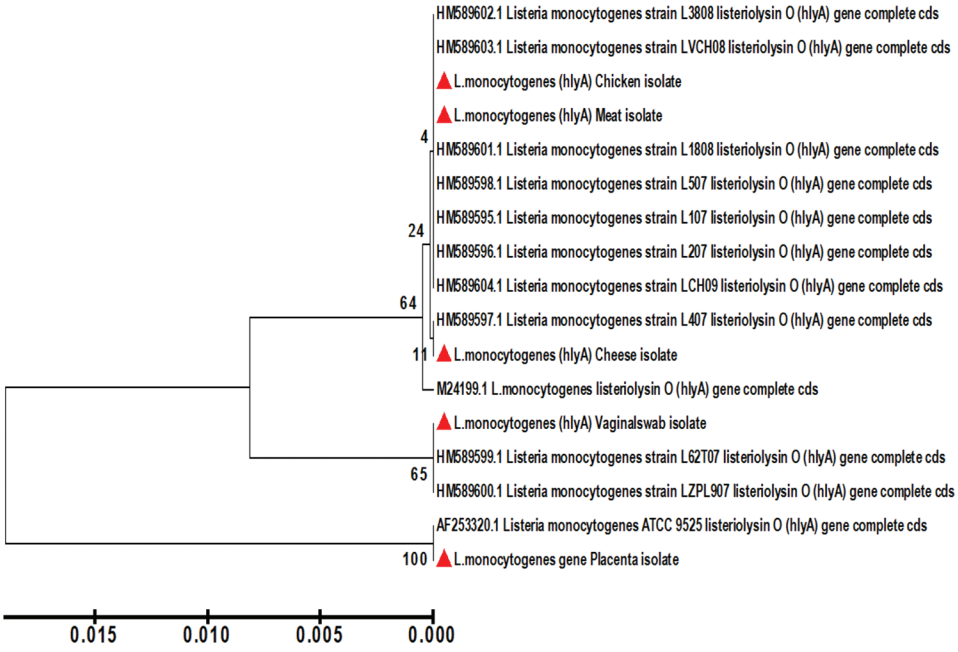

Fig. 2: The phylogenetic analysis based on *hlyA* gene showing relationship between Iraqi *Listeria monocytogenes* isolates (red triangle) with reference sequences from GenBank database, analyses were performed in MEGA6 software

Table 4: Details of *L. monocytogenes* isolates used in the present study with the accession numbers in Gene Bank from different source

No.	Source of isolates	Accession numbers
No. 1	Chicken meat isolate	MF688809
No. 2	Cheese isolate	MF688810
No. 3	Red meat isolate	MF688811
No. 4	vaginal swap isolate	MF688812
No. 5	Placenta isolate	MF688813

L. monocytogenes: Listeria monocytogenes

secondary vacuoles enabling it to invade other phagocytes, and this is considered one of its major virulence factors [22,23].

The phylogenetic tree analysis based on *hlyA* gene sequence has clearly shown that *L. monocytogenes* could be a reliable sign to indicate the presence of *L. monocytogenes*, which agrees with the study of Soni *et al*. [24]. In our current study, we identified five isolates of *L. monocytogenes* originating from clinical and food samples based on *hlyA* gene sequence.

Fig. 1 displays the differences in the sequences of the five Iraqi isolates (1–5) which we examined in comparison with the other Gene Bank isolates depending on the analysis of the *hlyA* gene.

Blasting the obtained sequences with those in database and the deduced phylogenetic analysis based on the *hlyA* gene (Fig. 2) of the Iraqi isolates showed that there is a significant genetic correlation between isolation No.1 have high homology with Indian isolate (HM589603.1), isolate No.2 with Indian isolate (HM589597.1), isolate No. 3 with US isolate

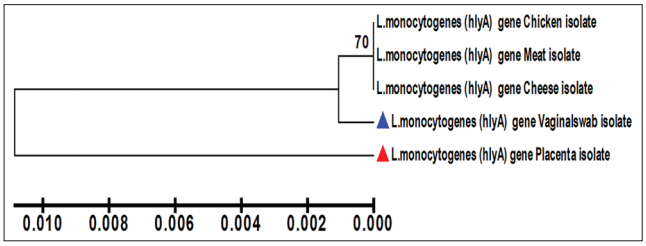

Fig. 3: The phylogenetic analysis for the 5 *Listeria monocytogenes* isolates in this study showed a clear genetic affinity in the isolates of *L. monocytogenes* isolated from chickens, meat, and cheese more than the isolates of *L. monocytogenes* isolated from the placenta and vaginal swabs

(M24199.1), isolate No.4 with Indian isolate (HM589599.1), and isolate No.5 with Italian isolate (AF253320.1).

Moreover, in Fig 3, the genetic analysis for the 5 L. *monocytogenes* isolates in this study showed a genetic affinity in the isolates of *L. monocytogenes* isolated from chickens, meat, and cheese by 70%, while there is a genetic difference in the isolates of *L. monocytogenes* isolated from the placenta and vaginal swabs.

CONCLUSION

The *hlyA* gene sequence information could be the reliable option to indicate the presence of *L. monocytogenes*, tracking the source of infection in human, the quality of foods, and frozen food and identifying the geographical distribution. More work, however, is required on *L. monocytogenes* to ascertain its presence in clinical and non-clinical samples in Iraq.

AUTHORS CONTRIBUTION

Maitham Ghaly Yousif and Ataa Khalil AL-Shamari: Biology Department Collage of science University of Al-Qadisiyah, Iraq.

CONFLICTS OF INTERESTS

None.

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