Analysis of hlyA gene sequencing of Listeria monocytogenes isolated from Iraqi women with recurrent miscarriage

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Abstract

Listeria monocytogenes is a causative agent of listeriosis and is regarded as one of the main foodborne pathogens. The transfer of L. monocytogenes to pregnant women occurs may cause miscarriage, stillbirth, or premature birth of the infected baby. This study was performed between November 2019 and June 2020, including 176 women with recurrent miscarriages at 17-46 years old who attended some Baghdad hospitals, in Iraq. The specimens were cervical swabs. Listeria agar was used for the isolation of L. monocytogenes and the isolates were identified with VITEK 2 system. The polymerase chain reaction was used for 16S rRNA and virulence genes (hlyA and inlA). The detection of variations of the hlyA gene sequence was done by using suitable software (MEGA6 and BioEdit). The recurrent miscarriages women with Listeria monocytogenes infections was 57 (32%) out of 176 aborted women and most aborted women with L. monocytogenes infection had previous abortions within the first trimester. The PCR products showed that 16S rRNA, hlyA, and inlA genes were detected in all identified isolates (100%). The results of alignment of the hlyA gene sequence revealed high similarity among the local isolates with strains reported from different parts of the world available in the public database, GenBank, on the other hand, the isolates L2 and L11 exhibited many variations at different positions of the gene sequence. It was found a high identity and phylogenetic relationship of the studied hlyA gene from 7 L. monocytogenes local isolates with those in European countries, Canada, and the USA which was found in strains isolated from foods and different infections. In conclusion, the detection of the virulence genes (hlyA and inlA) in addition to the housekeeping gene 16S rRNA, by PCR establishes the confirmation of L. monocytogenes identification, also some of the isolates may have variations that contribute to the severity of Listeria infections.

Keywords: L. monocytogenes, hlyA gene, abortion, Sequencing.

Introduction

Listeria monocytogenes is a Gram-positive facultative anaerobic, ubiquitous, and persistent bacterium in food processing plants because of inadequate hygiene and manufacturing practices (Amajoud *et al.*, 2018). This species causes listeriosis, a disease characterized by low morbidity but high mortality in those who are infected, and the most at-risk groups are pregnant women, newborns, children, and older adults (Schlech, 2019). The severity of *L. monocytogenes* infection is associated with several factors such as infecting dose, host immunity, and expression of virulence factors (adherence, invasion, immune modulator, intracellular survival, toxins, and mobile genetic elements) (Kwon *et al.*, 2020).

Pregnant women are particularly prone to infection as the placenta provides a protective niche for the growth of *L. monocytogenes* (Bakardjiev *et al.* 2005). In miscarriage, besides stillbirth, neonatal infection, severe necrotizing hepatitis, placental necrosis, and increased risk of postimplantation loss. Latent listeriosis in pregnant women leads to habitual abortions, intrauterine deaths, and fetal malformations (Abram *et al.* 2003). Many studies indicated the dominant and common serotypes of *L. monocytogenes* from spontaneous abortion and demonstrated that the presence of *hlyA* and other virulence genes were effective genes in increasingly aggressive and pathogenicity (Kaur *et al.*, 2007; Rezaei *et al.*, 2019). One of the Iraqi studies about the role of *L. monocytogenes* in abortion revealed that *L. monocytogenes* may have an obvious role in pregnancy loss and should be considered with spontaneous abortion (Hassan *et al.*, 2020).

The present study aims to investigate the distribution of virulence genes among *L. monocytogenes* strains isolated from Iraqi women with recurrent miscarriages, and also investigates the variation and mutations in the *hlyA* gene as the main virulence gene related to abortion among the local isolates.

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Materials and Methods

Isolation and identification of L. monocytogenes

This study was performed at Hospitals in Baghdad, Iraq, between November 2019 and June 2020. Out of 176 cervical specimen swabs were collected from patients with recurrent miscarriages. HiCrome™ Listeria Agar Base was used as both a selective and differential agar medium for quick and direct recognition of *Listeria monocytogenes*. The biochemical tests were achieved for confirmation identification, in addition to, VITEK 2 system (bioMerieux, France), according to the manufacturer's recommendations.

DNA extraction and identification of LM genes by PCR

Bacterial DNA was extracted from all *L. monocytogenes* isolates using ready-to-use kit (Promega, USA). The purity of the isolated DNA was monitored by NanoDropper 2000 (Thermo Scientific, USA). The PCR reactions for the detection of *L. monocytogenes* genes were done within a total volume of 20 µL. The reaction mixture contained Master Mix 10 µL, extracted DNA 3 µL as a template, and 0.7 µL of forward and reverse primers, then Nuclease free water 5.5 µL. The Primer sequences used for the detection of *L. monocytogenes* genes in this study were as in Table 1.

Table 1: Primer sequences for PCR detection of virulence and housekeeping genes in L. monocytogenes

Target gene	Oligonucleotide primer sequence (5'→3')	Size of product (bp)	References
hlyA	F: ATACCACGGAGATGCAGTGAC R: TCTTCTTGCATTTTCCCTTCAC	510 bp	(Mohammed, 2019)
intA	F: AGTGCGCTTTCAGGTTTAACT R: AAACTCGCCAATGTGCCTAT	278 bp	(Pangallo et al. 2001)
16S rRNA	F: CCTACGGGAGGCAGCAGT R: CGTTTACGGCGTGGACTAC	475 bp	(Chiang et al., 2006)

For amplification of LM genes, PCR conditions were carried out by the thermocycler (Applied Biosystems, Malaysia) according to the conditions of the previous studies mentioned above. Agarose gel electrophoresis was done with a 2 % Agarose Gel at 80V for 1.5 hour. After electrophoresis fragments were stained with Safe Green Dye and then visualized with ultraviolet light.

Sequencing analysis of hlyA gene

Seven selected *L. monocytogenes* isolates were selected to conduct the nucleotide sequence of *hlyA* genes. 20 µl of each PCR product of each gene were stored at -20˚C until used then the sequencing was carried out by Macrogen DNA Sequencing (Seoul, Korea). The nucleotide sequences of the *hlyA* genes of *L. monocytogenes* reference strains reported from different parts of the world (available in public database: Gen Bank) were downloaded and aligned using the ClustalW method of the MEGA6 program. The detection of variations in the gene sequence and the translation of nucleotides to amino acids were done by using suitable software (MEGA6 and BioEdit).

Results and Discussion

The present study included 176 aborted women with recurrent miscarriages. The results of isolation and identification revealed that 32% of the aborted women were infected with *L. monocytogenes* according to the findings of the selective HiCrome™ Listeria agar and VITEK 2 system. These identified isolates were included with the molecular identification and detection of the virulence genes.

Genomic DNA extraction

Using a genomic DNA purification kit (Promega, USA), Genomic DNA was extracted from L. monocytogenes isolates. Extraction of genomic DNA from 57 isolates was confirmed as bands by gel electrophoresis. The results of DNA extraction were shown in Figure (1). DNA concentration and purity were measured by a Nanodrop spectrophotometer, all the isolates had DNA concentrations between (50-100 ng/µl) and the purity of the DNA was (1.4- 2).

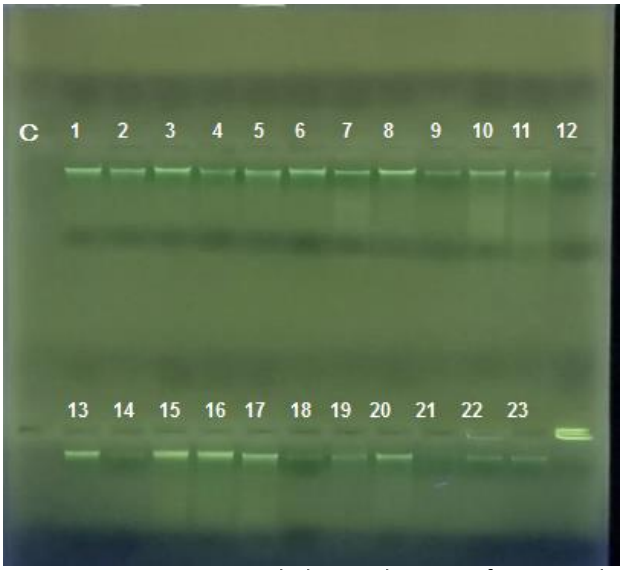

Figure (1): Agarose gel electrophoresis of extracted DNA to check purity and integrity. Lane 1-23: DNA of different L. monocytogenes isolates, Lane C: Negative control (70 V/ 30 min.).

Molecular identification of Listeria species by detection of the 16S rRNA gene

The PCR results (Figure 2) showed that the *16S rRNA* gene (475 bp) exists in all 57 *L. monocytogenes* identified by the previous identification methods, and this confirmed the accuracy of our tests and methods used for the identification of this genus.

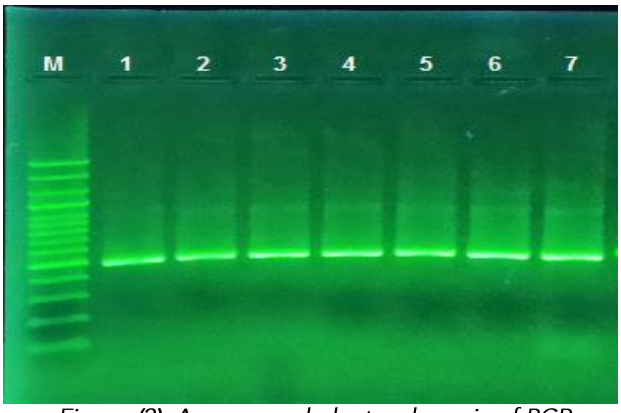

Figure (2): Agarose gel electrophoresis of PCR amplified products for 16S rRNA gene. Lane (M): l00 bp ladder, (80 V for 1.5 hr). Lane (1-7): positive result with positive bands of 475 bp L. monocytogenes.

Molecular detection of the *16S rRNA* gene is one of the most commonly used for bacterial identification, where, the *16S rRNA* gene sequencing is too conserved, and is not polymorphic enough to differentiate all *Listeria* spp. (Srinivasan *et al.*, 2015). The present findings suggested that PCR using the *16S rRNA* gene was an excellent method for the detection of *Listeria* spp. isolates. These results are in agreement with other studies which mentioned that the detection and sequencing of this gene is an effective means for the identification of clinical isolates of *Listeria* (Somer *et al.*, 2003; Kumar *et al.*, 2015).

Detection of virulence genes by Polymerase Chain Reaction (PCR)

In order to detect the presence of *L. monocytogenes* virulence genes (*hlyA* and *inlA*) and determination, the prevalence of each gene among *L. monocytogenes* clinical isolates, uniplex polymerase chain reaction (PCR) for each DNA extracted sample has been used. The PCR reaction included 57 isolates for the detection of the genes. The PCR products have been confirmed by analysis of the bands on gel electrophoresis. PCR products have been confirmed by comparing their molecular weight with 100 bp DNA Ladder. The results of uniplex PCR reaction for *hlyA* and *inlA* genes showed in Figures (3), and (4).

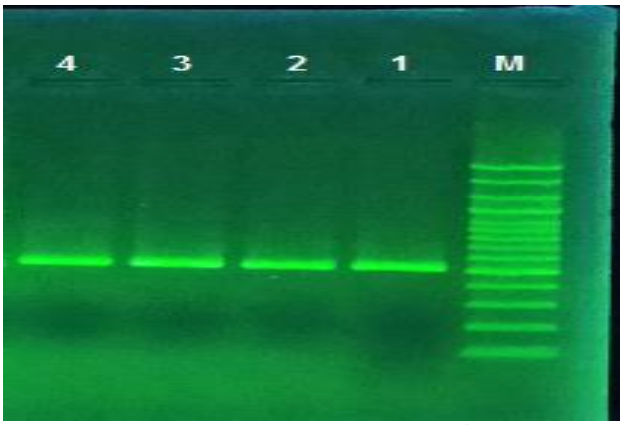

Figure (3): Agarose gel electrophoresis of PCR products for the virulence gene hlyA (510 bp). Lane M: 100 bp DNA ladder; lanes 1-4: L. monocytogenes (80 V for 1.5 hr).

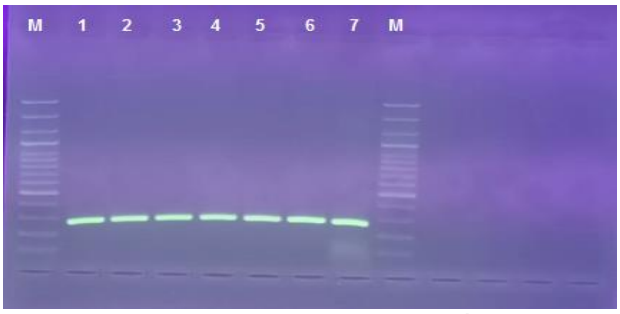

Figure (4): Agarose gel electrophoresis of PCR products for the virulence gene inlA (278 bp). Lane M: 100 bp DNA ladder; lanes 1-7: L. monocytogenes (80 V for 1.5 hr).

The results of PCR reaction for detection *hlyA* gene (Figure 3) revealed the presence of *hlyA* genes in all 57 (100 %) *L. monocytogenes* clinical studied isolates. These results reflected the more accurate and more sensitive detection of molecular diagnosis in comparison with HiCrome™ Listeria Agar Base and biochemical tests. The results were in agreement with most previous studies which indicated the presence of this gene in all clinical isolates of *L. monocytogenes* and not detected among any other *Listeria* spp., consequently, the *hlyA* gene is normally considered to be species-specific to *L. monocytogenes* (Burall *et al.*, 2011). The PCR detection of *L. monocytogenes* largely depends on the selection of an appropriate target gene, and many studies demonstrated that *hlyA* gene was the suitable gene for molecular identification, which encoded Listerolysin O (LLO) (Aznar *et al.*, 2003; Nguyen *et al.*, 2004).

In the present study, the *inlA* gene was detected in all *L. monocytogenes* isolates (100%); these results were in agreement with those obtained by Liu *et al.* (2007), which indicated that the use of the multiplex PCR targeting *inlA, inlC, and inlJ* genes contribute with the confirmation of *L. monocytogenes* species identity and virulence.

Several studies showed that the *inlA* gene is species-specific, and also the virulence factors, including hemolysin (*hlyA*), invasive associated protein (*iap*), and internalin A (*inlA*), is necessary for the pathogenesis of *L. monocytogenes*. Therefore, detection of multiple virulent genes in PCR assay is desirable as it reduces the time and will be useful for large-scale investigations for the detection of pathogenic strains of *Listeria* (Rawool *et al.*, 2007; Abdeen *et al.*, 2021).

Aligning the obtained sequences (7 isolates L1 to L6 and L11) with the reference strains in GenBank confirmed the correct identification of *hlyA* genes by PCR. Also, these sequences were analyzed for the presence of variants of these genes and the detection of the differences in the nucleotides (mutations).

The results of the alignment of the *hlyA* sequences of the local isolates No.1 with the reference strain NRRL 33090 isolated from an animal with accession number AY512437 (USA) (Figure 5); revealed that the sequence of *hlyA* gene of the local isolate *L. monocytogenes* had high similarity (99 %).

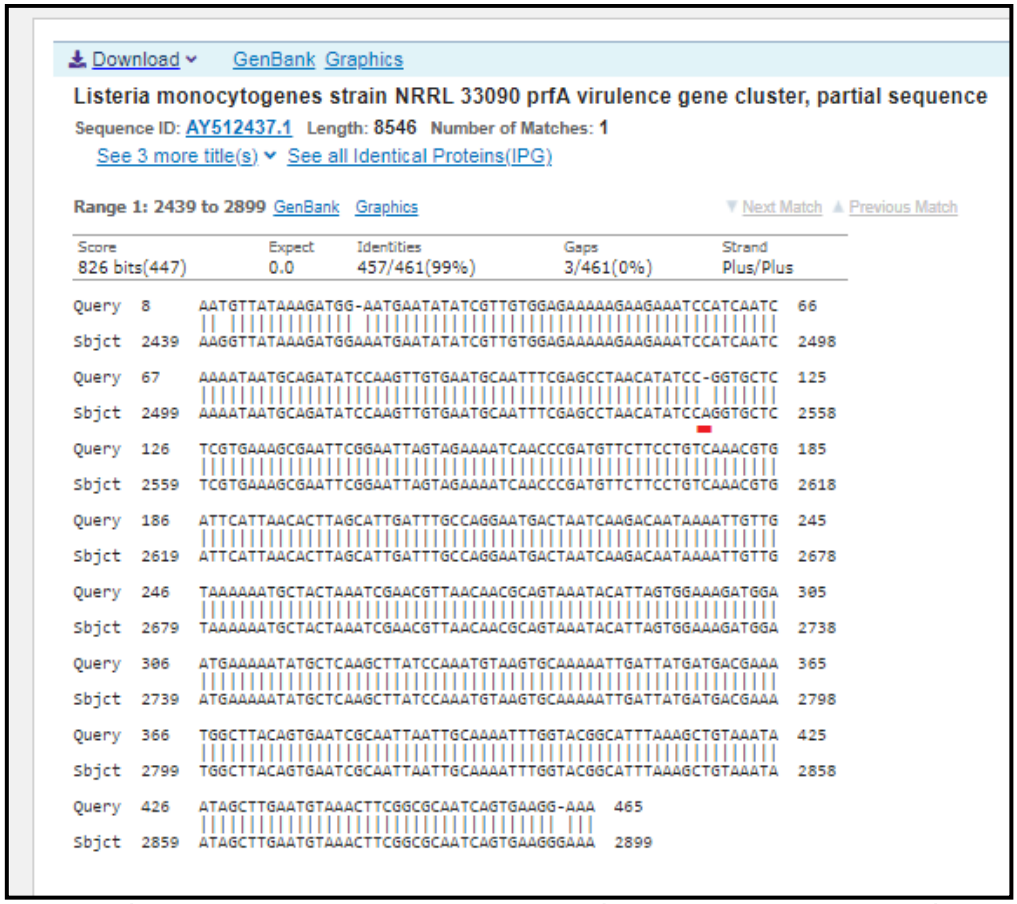

Figure (5): Alignment of L. monocytogenes hlyA gene sequence of the local isolate No.1 with reference strain (NRRL 33090) available in GenBank.

The results of the alignment of the *hlyA* sequences of the local isolates No.2 with the reference strain NRRL 33090 isolated from an animal with accession number AY512437 (USA) (Figure 6), demonstrated that the sequence of *hlyA* gene of the local isolate *L. monocytogenes* No.2 had many variations of the nucleotides which included different types of mutations such as transitions and transversions and this obvious in our query at the positions such as 79, 82, 111, 117 and 129, also in other positions as showed in (figure 6).

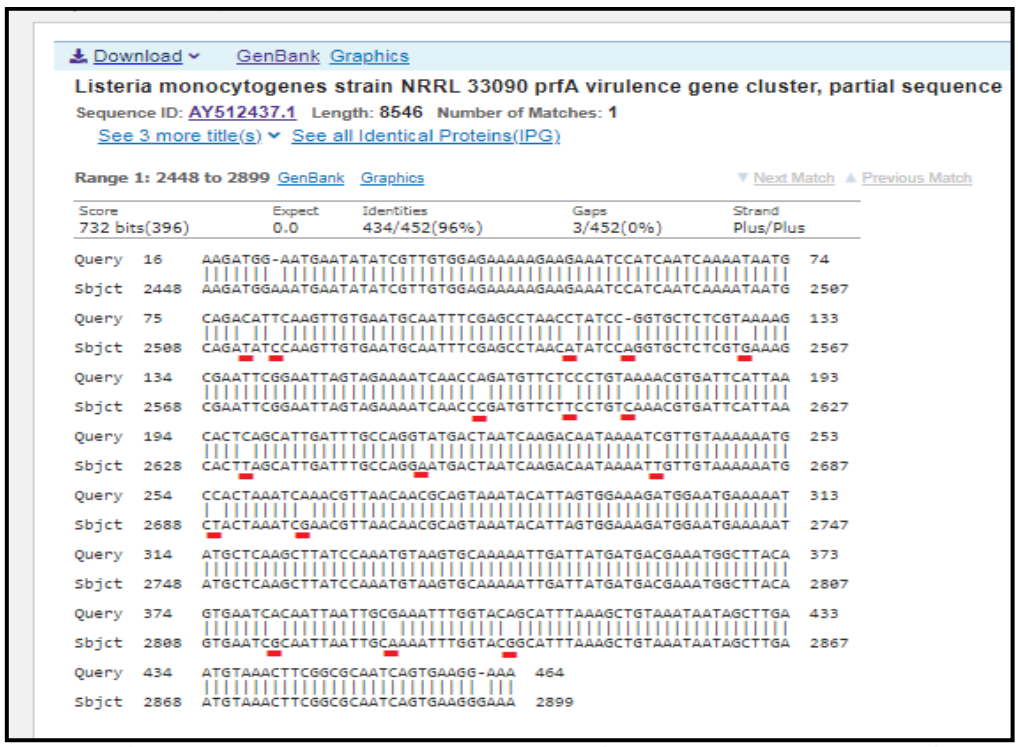

Figure (6): Alignment of L. monocytogenes hlyA gene sequence of the local isolate No.2 with reference strain (NRRL 33090) available in GenBank.

The results of phylogenetic analysis of the *hlyA* gene from 6 local isolates with the *hlyA* reference genes from The NCBI GenBank database were demonstrated in (figure 7). Single nucleotide polymorphisms (SNPs), one of the dominant forms of evolutionary change, have become an indispensable tool for phylogenetic analyses (Faison, 2014). The classification based on the nucleic acid sequence, an outcome of molecular analyses of phylogenetic markers, has become more popular, where it was found that rRNAs with the mosaic structure composed of a succession of several domains that are more or less conserved as a result of variable evolution rates, makes such studies relatively more useful (Collins *et al.*, 1991).

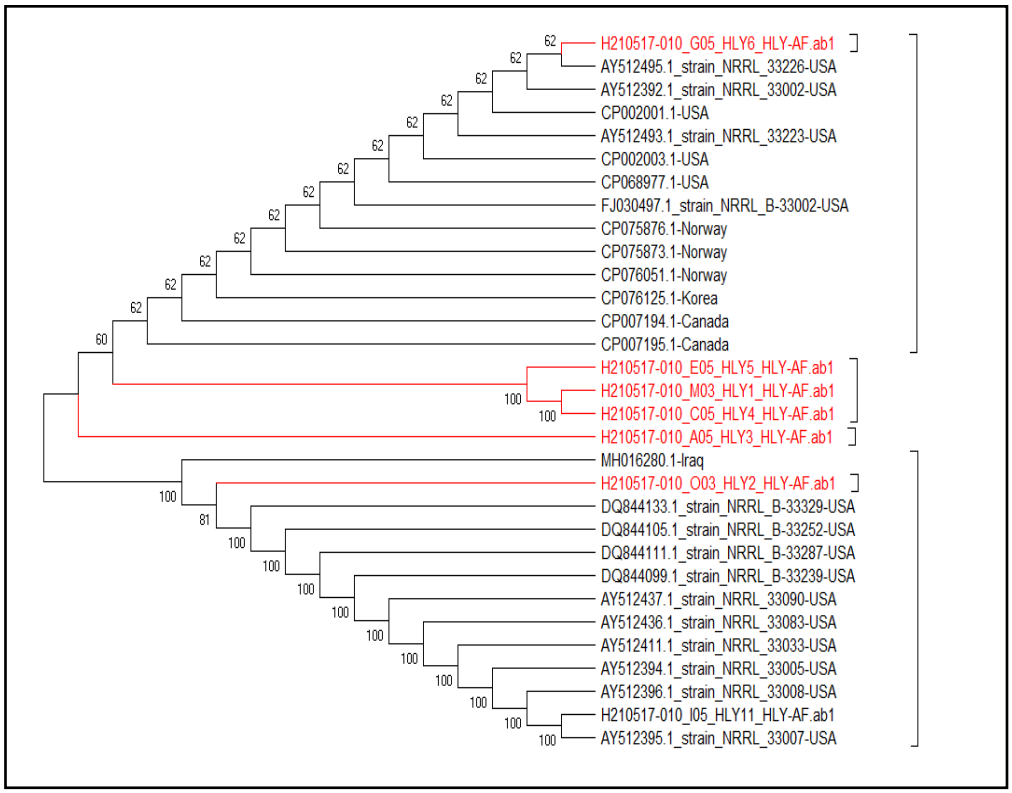

Figure (7): Phylogenetic relationships based on the partial nucleotide sequence of the hlyA genes of L.monocytogenes local isolates (L1-L6). Cluster analysis was based upon the UPGMA (Unweighted Pair Group Method with Arithmetic mean) method.

All of our isolates were placed in the two clusters, L2 in one cluster which had high similarity with the local strain from Iraq with accession number (MH016280.1) and global strains from the USA, and the rest isolates in the other. The clusters also included *L.monocytogenes* species reported from elsewhere. The phylogenetic relationships analysis of the 5 local isolates L1, L3, L4, L5, and L6 which had many differences in their nucleotides, demonstrated a high similarity with many isolates especially those isolated from foods, and systemic infections in the USA, Norway, and Canada. Also, the phylogenetic tree observed 100% identity of the local isolates sequence L1, L4 andL5, which was found in the same cluster of the isolates L3 and L6.

A previous study revealed the phylogenetic tree based on *hlyA* gene sequence clearly differentiates between the *L. monocytogenes*, *L. ivanovii*, and *L. seeligeri*. And the identification of 80 isolates of *L. monocytogenes* originating from different clinical, food and environmental samples based on *16S rRNA*, and *hlyA* gene sequence similarity (Soni *et al.*, 2014).

In Korea, two housekeeping genes, of *Listeria monocytogenes dat* and *hlyA*, were analyzed in a set of 28 isolates from different sources to estimate their genetic diversities, and there was a good correlation between the PFGE patterns and phylogenetic grouping of two gene sequences among the isolates (Kyun, 2005).

In Iraq, the phylogenetic analysis of *hlyA* gene according to the sequence information could indicate the presence of *L. monocytogenes*, tracking the source of infection in humans, foods, and frozen food and identifying the geographical distribution (Yousif and Al-shamari, 2018).

Conclusion

The present study showed that the molecular methods for detection of the virulence genes related to the species *L. monocytogenes*, and support this detection by the sequence, especially with *hlyA* gene, can be effective in the accuracy and specificity of identification with finding the variations of the local isolates.

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