Detection of *Listeria monocytogenes* in Several Types of Frozen Meat in Baghdad city

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Abstract
Detection of pathogenic bacteria, such as *Listeria monocytogenes*, in food is crucial for safeguarding public health in Iraq. Forty five samples of frozen meat (15 samples of each of minced red meat, chicken, and fish) were collected from different markets in Baghdad city. Molecular (RT-PCR) and culturing (conventional microbiological examination) methods were used to determine the level of contamination of *L. monocytogenes* in these types of meat.

For the culturing method, TSYEB broth was used as an enrichment medium, whereas BALCAM medium (HiMedia) with the listeria selective supplement FD061 was used as a selective medium, for the isolation and identification of this bacterium. The isolates were confirmed microscopically and biochemically. The results of the culturing method showed that the total number of the isolates of *L. monocytogenes* was 14/45 (31.1%). The incidence of this bacterium was high in fish (11/15, 73.3%), while it was low in the other two types of meat. 2/15 (13.3%) in red meat and 1/15 (6.7 %) in chicken.

Molecular detection of each sample of the bacteria was performed using RT-PCR technique after preparing the Genomic DNA extraction of these samples according to the protocol provided by ReliaPrep™ Blood gDNA Miniprep System kit (Promega, USA). The PCR primers and the hybridization probe ((Macrogen, Korea) were used to target the *ina* gene sequence (specific for *L. monocytogenes*). The results of the RT-PCR assay showed that 10/45 (22.2%) of the samples were positive for *L. monocytogenes*, which was detected only in fish samples ((10/15, 66.7%), while not found in minced red meat and chicken. However, our results showed differences when compared to other previous works because there were many studies found that the highest contamination rate was in red meat products.

We conclude that the PCR kit used for the detection of *L monocytogenes* appears to give accurate results in the diagnoses of this bacterium in meat products and in comparison with the other routine diagnosis methods in the laboratory, which included culturing and doing biochemical tests which last for approximately 7 days, the RT-PCR technique was able to confirm the findings within 48 hours.

**Keywords**: Isolation; *Listeria monocytogenes*; Real time polymerase chain reaction; frozen meats; Baghdad
Introduction

Listeria monocytogenes is a pathological, Gram-positive, facultative, intracellular bacterium that has the capacity of causing serious illnesses in animals as well as humans [1]. It is associated with a spectrum of diseases in humans, such as meningocencephalitis, septicemia, and abortion, especially in the more vulnerable parts of the population, including pregnant women, infants, and geriatric and immunocompromised people. In addition, L. monocytogenes can cause mild to severe febrile gastroenteritis, which is a noninvasive illness in adults. However, in persons with weak cell-mediated immunity, Listeriosis may lead to meningitis, being an uncommon cause of this disease in adults [2].

The importance of pathogenicity of L. monocytogenes is based on the ability of this bacterium to survive and multiply in phagocytic host cells. Apparently, it can invade the gastrointestinal epithelium as an intracellular infection by the action of a protein that is called internalin (InlA/InlB), which allows the bacteria to attach to the cadherin protein on the intestinal cell membrane through a zipper mechanism [3]. L. monocytogenes strains differ in the number of internalin genes encoded in their genomes [4]. Null mutations in four internalin genes (inlA, inlB, inlC, and inlJ) resulted in reduced invasion or virulence in tissue cultures or animal models [5].

L. monocytogenes is widely distributed in nature, as it is recognized as a primary inhabitant of the soil and decomposing vegetation [6]. It is able to grow at acidic pH, high concentrations of NaCl, and cold environment, as well as being considered as a psychrotrophic species [7]. These, beside other factors, conferred a great importance to this species in the food industry sector [8].

Listerial infections were reported to be increased, mainly those associated with food consumption, such as meals ready-to-eat (MRE) [9-12]. Even some epidemiologically monitored flare-ups were recorded [13], leading this bacterium to be declared as a main food-borne pathogen.
**L. monocytogenes** is a major concern to the public health and food industry, because of its prevalence in the environment and adaptability to survive or even thrive under very harsh circumstances [14]. The increasing rate of **L. monocytogenes** contamination, especially in modern eating style, has led to the rapid action of the discovery of new and fast methods for testing food products.

Almost all listeriosis cases are foodborne. A high variety of foods could be contaminated by **L. monocytogenes**, including raw poultry meat, ground beef, soft dairy products, and fish. Most of these items are widely consumed in Iraq [15].

Many bimolecular methods and techniques were established for the diagnosis of **L. monocytogenes**, including DNA probes and PCR (polymerase chain reaction) techniques [16, 17]. In addition, a direct method for the detection of **L. monocytogenes** in food products by PCR was recorded in many studies [18].

Because of high sensitivity, specificity, and low time consumption of PCR, this and other molecular methods have been recommended [19-21]. However, the sensitivity and specificity of the PCR are dependent upon many factors, such as DNA extraction procedures, the target genes, and the primer sequences [22]. In addition, a PCR assay can only determine the presence or absence of bacteria rather than the quantity of bacterial cells. Thus, the real-time PCR (RT-PCR) is recommended, which can adapt to any specific gene sequence used in the conventional PCR method [23].

The current study was designed to detect **L. monocytogenes** in different types of meat products by the molecular detection method (RT-PCR) in comparison with the routine diagnosis method (microbiological examination) in order to investigate the level of contamination in these types of food in Baghdad city.

**Materials and methods**

This study was conducted on a set of some types of frozen meat samples that were purchased from the local markets of different areas of Baghdad city. In a total of 45 samples, three types of frozen meat distributed as: 15 minced red meat, 15 chicken, and 15 fish (three different brands of each type and five replicates for each brand) were investigated. These brands (e.g., Al-Hasanat minced red meat, AL-Baraka minced red meat, Al-Halal minced red meat, Al-kafil chicken, Al-dur al abiad chicken, Al-sadia chicken, Alo-Sea fish, Al-Fakhir fish, and Carp fish) were collected randomly during the period from June to July, 2019. These samples were tightly sealed by polyethylene bags.

**The isolation of **L. monocytogenes**:**

Frozen meat samples were transferred to the laboratory and 1g of each sample was thoroughly homogenized. The samples were preserved in 20ml of TSYEB broth (tryptic soya yeast extract), as an enrichment medium, at 4°C for five days (cold incubation method) in order to reduce other bacterial contamination, because only listeria can grow at low temperatures, overgrowning other organisms which grow more slowly if at all [24, 25]. Then, the growth was initially streaked on listeria agar (Balcam /HiMedia, India) supplemented with listeria selective supplement FD061 containing Polymyxin B sulphate, Cefazidime, and Acriflavine hydrochloride [26]. The media was incubated at 37°C for 24 hours. After purification by sub culturing the bacteria, the pure isolates were examined microscopically by using Gram stain and tested by some biochemical tests, such as catalase, oxidase, sugar fermentation, Esculin hydrolysis test and motility at 22°C [27]. Also, the blood haemolysis test was performed to confirm the diagnosis [28].

**Molecular detection using RT-PCR**

DNA extraction was conducted for each sample of meat and genomic DNA was isolated according to the protocol of ReliaPrep™ Blood gDNA Miniprep System (Promega, USA). The set of PCR primers and the hybridization probe (Macrogen, Korea) were appropriate for this work, as shown in (Table-1), in order to target the *ina* gene sequence specific for **L. monocytogenes** and the specificity of these primers as well as the hybridization probe were verified according to a previous work [29].

Run started on (Mic qPCR Cycler, Bio Molecular System, Australia) Serial no. "mic M0000336" S/W, v2.4.0.

During this work, a positive control was used in order to optimize RT-PCR technique and to match with the PCR primers and hybridization probe. This control was represented by a DNA extract of **L. monocytogenes** (accession numbers MH092995.1) confirmed previously by PCR and DNA.
sequence obtained from the BLAST-N program (National Center for Biotechnology Information) and recorded by a previous study on carp fish sample [30].

Table 1- Primers and hybridization probe used in this study.

| Name   | Purpose  | Sequence (5’ - 3’)              |
|--------|----------|---------------------------------|
| inlA-F | Forward primer | TCGCAAACAGATCTAGACACAGGT-3’   |
| inlA-R | Reverse primer | GTTCAAGTATTTCAATCCATCGATG-3’   |
| inlA-P | FAM- L. monocytogenes hybridization probe | FAM- CAACGCTTCAGCGGATAGATTAGGGAT-3’-TAMRA |

Annealing temperature (60°C)

Each RT-PCR assay was performed in a total volume of 10μl of the mixture (7 μl of Master mix and 3 μl of template). Master mix consisted of the forward and reverse primers, and template DNA. Thermal cycling was performed at 95°C for 5 minutes and 50 cycles as follows: 95°C for 15 s and 60°C for 1 minute.

Results

For the culturing method, BALCAM medium was used as a selective medium for the isolation of Listeria. Bacterial colonies appeared as greyish/black colonies with black halos (Figure-1). The bacteria was examined microscopically and showed gram positive rods, whereas some were arranged as V- and Y-shaped.

![Figure 1](image)

Biochemical tests were conducted for each isolate. The bacterium was catalase positive, oxidase negative, mannitol fermentation negative, esculin hydrolysis positive, and motile at 22°C. In addition, β-haemolysis test was positive.

The results of the culturing method of different types of frozen meats showed that, out of 45 samples, the total number of bacterial isolates of L. monocytogenes was 14 with a percentage of 31.1%. These isolates were distributed as 2/15 (13.3%) and 11/15 (73.3%) of minced red meat, chicken, and fish, respectively (Table-2).

The same samples were tested by RT-PCR and the results demonstrated that 10 (22.2%) were positive, being detected only in fish samples 10/15 (66.7%), whereas no isolates were found in minced red meat and chicken samples (Table-2). The cycle quantity (Cq) of fish samples ranged from 35.47 to 38.30, while that of the positive control sample was 11.12, as shown in Figure-2.
Table 2 - Results of detection of *L. monocytogenes* in different types of meat samples:

| Sample     | No. of Sample | Diagnoses by culturing | Amplifying by PCR |
|------------|---------------|------------------------|-------------------|
| Red meat   | 15            | 2 (13.3%)              | 0                 |
| chicken    | 15            | 1 (6.7%)               | 0                 |
| fish       | 15            | 11 (73.3%)             | 10 (66.7%)        |
| Total      | 45            | 14 (31.1%)             | 10 (22.2%)        |

Figure 2 - Cycle quantity (Cq) of positive samples and positive control as shown at amplification curves reported by the PCR program software.

Discussion

*L. monocytogenes* can be isolated from raw milk, meat, poultry, fish, vegetables, cheese, ice cream, and ready-to-eat (RTE) products [31-34]. Most of these items are widely consumed in Iraqi cities. This was found to be a serious public health problem because this bacterium can spread through the consumption of these products, causing different infections, including Human listeriosis [35].

Only few studies that are concerned with this field, especially in Baghdad, were published. Therefore, our aim was to detect the prevalence of *L. monocytogenes* in different types of frozen meat samples found in the markets of Baghdad city.

*L. monocytogenes* was diagnosed in the samples by the culturing method as well as by using a molecular assay (RT-PCR).

The results of the culturing method showed that 14/45 (31.1%) of the samples were positive for the growth of this type of bacterium, while 10/45 (22.2%) were subsequently identified as *L. monocytogenes* by using RT-PCR method. This bacterial isolate was detected only in fish samples 10/15 (66.7%), while not found in minced red meat and chicken samples.

During this investigation, no contamination was found by this type of bacteria in some raw frozen meat samples, such as minced red meat and chicken, in contrast to fish meat which is considerably more likely to be contaminated with *L. monocytogenes*. These results disagree with those reported by other studies conducted in Iraq [36, 37]. Our values also showed differences when compared to other studies performed in other countries. In Isfahan, Iran, a research was conducted on various food products, including dairy products, meat, and ready-to-eat food, and found a 4.7% contamination rate with *L. monocytogenes* [38], which is lower than that found by our study. However, the occurrence of *L. monocytogenes* in the current research was lower than that found in an earlier study [39]. Another research [40] found that the highest contamination rate was in red meat products.

Another explanation for the differences between our results and those of the previous works is that a wide range of animal species can become infected with *L. monocytogenes*, including mammals and domesticated animals [41]. However, cows are very rarely infected with this bacterium [42], in the opposite to sheep which is more sensitive to be infected with listeria [43]. In Baghdad markets, frozen
minced red meat is mostly from cow origin, which might be the reason for the negative detection of these bacteria in the types of food tested in this work. In addition, the low contamination may be due to the collection of the samples during the summer (hot season), which provides suitable conditions for the infection or the growth of listeria [44]. Another research conducted in Iraq found that the highest incidence of listeriosis occurred in the cold seasons [45]. Regarding chicken meat, our results showed the lowest incidence of L. monocytogenes compared with those found in other studies [46,47]. However, the numbers of samples of the previous studies were higher than those investigated in the current study.

L. monocytogenes cells that contaminate raw meats are likely to be damaged in some samples studied due to the effects of processing. In raw and processed meats, other bacteria, such as lactic acid bacteria, may become the dominant population and preserves the product from contamination [48]. This bacteria may cause competition for nutrients, production of toxic end products, or production of specific antibiotics against pathogenic bacteria like listeria [49].

A number of molecular biological methods were described for the detection of L. monocytogenes, including DNA probes and PCR techniques. Direct detection of L. monocytogenes in food products by PCR was reported in several cases [50-54].

Many studies described a qPCR assay for the specific detection of L. monocytogenes in food samples. Positive deviation was observed in ten analyzed samples. A possible explanation for these discordant results is that DNA from dead or viable, but non cultivable, L. monocytogenes cells was detected by the alternative method in the food matrix [55, 56]. Furthermore, negative deviation was not detected, which demonstrates the robustness of the alternative method, as food components such as organic compounds, calcium ions, glycogen, and lipids were demonstrated to inhibit PCR [57].

One to two stages of enrichment processes are used in the real-time PCR detection; these processes utilize different selective media. These processes are used by different enrichment and DNA preparation approaches in order to achieve increasing numbers of live cells to be in detectable level, decrease the numbers of dead L. monocytogenes cells, and reduce the foodborne PCR inhibitors [58].

Detection of L. monocytogenes in food by the conventional cultivation method involves the growth in a pre-enrichment medium, then on a selective medium, followed by a series of biochemical and serological tests to confirm the diagnoses [59]. These work-intensive methods are time-consuming as they may require up to 10 days to be finished. Real-time (RT)-PCR is rapid and allows an accurate identification and precise nucleic acid sequences quantification [60, 61]. The lack of post-PCR steps minimizes the cross-contamination risk and allows for a high output [62, 63].

To our knowledge, there are few published data regarding the prevalence of L. monocytogenes in food samples, especially frozen meat, in Baghdad areas. In fact, L. monocytogenes is rarely tested in food products by using PCR. Therefore, this work was conducted to assess the use of PCR for the direct detection of L. monocytogenes in meat products and determine the level of contamination in Baghdad city.

L. monocytogenes was diagnosed successfully by RT-PCR technique in some types of meat samples. The use of the kit designed specifically to the target inlA gene (internalin gene), including primers and hybridization probe, provided satisfactory results for the detection of this bacterium. In comparison with the routine diagnosis methods, including culturing and biochemical tests which last for approximately 7 days, the RT-PCR technique was able to confirm the findings within 48 hours.

The RT-PCR assay had high sensitivity and specificity and it can provide quick and reliable results for the diagnosis of a large number of foodborne pathogens that contaminate various types of food products. Improved methods for the diagnosis of the bacteria in food need to be available, including those based on the use of DNA probes, or the polymerase chain reaction, in order to prevent and control human infections.

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