

## ISOLATION AND CHARACTERIZATION OF *LISTERIA MONOCYTOGENES* FROM SOME IRAQI MISCARRIAGE WOMEN

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### ABSTRACT

This study was aimed to find out how common *Listeria monocytogenes* in a group of Iraqi women who had miscarriages. Also, investigates the dependability of molecular diagnosis, specifically evaluating the target *hlyA* gene as an alternate solution tool for identification. A total of 200 specimens comprising cervical swabs were collected from 176 women suffered from miscarriage and 24 women as control. The detection of *L. monocytogenes* in miscarriages women by using HiCrome™ *Listeria* Agar Base as both a selective and differential agar medium for quick and direct recognition of *Listeria* species. The biochemical tests were achieved for confirmation identification. In addition, virulence-associated genes (*hlyA* gene) isolates were identified using a Real-time PCR approach. Results show out of the 200 (patients and control) specimens, 176 women with miscarriages were analyzed and it was found that 117 (66.5%) specimens from abortion cases were negative for *L. monocytogenes*, while 59 (33.5%) specimens were positive for *Listeria* species. All 24 women of control give negative result. Seventy-five (32.4%) isolates from the 59 positive women showed the presence of the *hlyA* gene in Real-time PCR. We found that molecular diagnostic through Real-Time PCR is a quick and accurate diagnostic method that can help minimize the time it takes to start a specific treatment and track its success.

Keywords: abortion, listeriosis, *hlyA* gene, Real-Time PCR.

هبة وآخرون

مجلة العلوم الزراعية العراقية- 328-322:(1)55:2024

عزل وتوصيف الليستريا المستوحدة من بعض النساء العراقيات المجهضات

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### المستخلص

هدفت هذه الدراسة إلى تحديد نسبة الإصابة بـ *Listeria monocytogenes* في مجموعة من النساء العراقيات المجهضات. تبحث هذه الدراسة في موثوقية التقنيات الجزيئية ، وعلى وجه الخصوص تقييم جين *hlyA* المستهدف كأداة بديلة لتحديد *L. monocytogenes* ولتقييم ارتباط هذا النوع بالإجهاض في مجموعة من النساء العراقيات. تم جمع 200 عينة من مسحات عنق الرحم من 176 من النساء اللاتي يعانين من الإجهاض و 24 امرأة كسيطرة. تم الكشف عن هذه البكتريا في حالات الإجهاض باستخدام أكار الليستريا الاساس <sup>TM</sup> HiCrome كوسط أكار انتقائي وتشخيصي لتحديد سريع ومباشر لأنواع الليستريا. تم إجراء الاختبارات البيوكيميائية لتأكيد تحديد وجود هذه البكتريا. كذلك تم إجراء الكشف الجزيئي باستخدام تقنية تفاعل البوليميراز المتسلسل في الوقت اللحظي لتحديد الجين المرتبط بالفوعة (*hlyA*) في عزلات البكتريا. من بين 200 (نساء مجهضات و سيطرة) ، تم تحليل 176 امرأة مجهضة ووجد أن 117 (66.5%) عينة من حالات الإجهاض اعطت نتيجة سلبية لوجود *L. monocytogenes* ، بينما 59 (33.5%) عينة من حالات الإجهاض كانت إيجابية. في حين كل مجموعة السيطرة 24 امرأة اعطت نتيجة سلبية. أظهرت 57 (32.4%) من بين 59 امرأة إيجابية لوجود جين *hlyA* في تفاعل البوليميراز المتسلسل في الوقت اللحظي. الخلاصة اشارت إلى أن التشخيص الجزيئي باستخدام تفاعل البوليميراز المتسلسل في الوقت اللحظي هي طريقة تشخيص سريعة وموثوقة تساهم في تقصير الوقت اللازم لبدء العلاج ومراقبة فعالية العلاج لحالات الاجهاض.

الكلمات المفتاحية: إجهاض، داء الليستريات، جين *hlyA*، الوقت اللحظي PCR

Received: 13/1/2022, Accepted:23/3/2022

## INTRODUCTION

Listeriosis is caused by *Listeria monocytogenes*, the Gram-positive bacteria which is an opportunistic infection in humans and several animal species. Listeriosis is a life-threatening infection affecting humans, with a mortality rate of about 20% (14). *L. monocytogenes* is an animal pathogen causing infectious diseases. It was found that this species was one of the agents responsible for abortions in infected cows (15). Also, *Listeria monocytogenes* were identified and isolated from contaminated food and contribute to food poisoning (8). This is a foodborne disease that can cause complicated infections in immunocompromised individuals, embryos, and neonates (5). Pregnant mothers account for 20–30% of listeriosis occurrences, according to studies. Listeriosis can cause bacteremia, amnionitis, and infections of the embryo leading to premature birth, miscarriage, stillbirth, and other serious health concerns for newborns (13). The molecular identification of *L. monocytogenes* was conducted by several virulence genes. These genes include the internalins, listeriolysin O, phosphatidylinositol-phospholipase C, actin (*act A*), *iap* (invasion associated protein), and virulence regulators (encoded by *prf A*). Infections and pathogenicity are aided by several virulence factors (11). *L. monocytogenes*, *Toxoplasma gondii*, rubella, herpes simplex virus (HSV), measles, and cytomegalovirus are among the diseases that have been linked to sporadic spontaneous pregnancy loss. The following mechanisms have been proposed for infectious causes of pregnancy loss: a) direct infection of the fetus, uterus, or placenta, b) amnionitis, c) placental insufficiency, d) an infected intrauterine device, or e) chronic endometritis or endocervicitis (7). A meta-analysis study showed there is a significant relationship between the infection with *L. monocytogenes* and spontaneous abortion (20). In Iraq there is a little prevalence or incidence data about the listeriosis infections and their correlation with recurrent abortion in Iraqi pregnant women, therefore this study was aimed to investigate the prevalence of *L. monocytogenes* in a group of Iraqi women with recurrent abortion and identification of

this bacterium by a molecular assay using the virulence gene (*hly*).

## MATERIALS AND METHODS

**Samples collection:** The cervical Swab specimens were collected using sterile transport swab medium as follows: 1- From patients with miscarriage, the swab specimens were collected after two to four weeks from the last abortion. 2- Healthy (control) women, the swab specimens were collected before or after the menstrual period. These clinical specimens of patients and control groups were collected from public maternity hospitals in Baghdad city during the period from November 2019 to December 2020.

### Bacterial culture media

The culture media used in this study included Brain Heart Infusion Broth M210 (BHI, Himedia), HiCrome Listeria Selective Supplement FD181, and HiCrome™ Listeria Agar Base, Modified M1417 (HiMedia, India). All media were prepared according to manufacturing instructions. The pH was adjusted to 7.3 ( $\pm 0.2$ ) and sterilized in an autoclave at 121 °C for 15 minutes at 15 pounds per square inch (PSI).

### *Listeria monocytogenes* isolation

*L. monocytogenes* was isolated from clinical specimens. The specimen swab was firstly inoculated in an autoclaved and sterilized universal containing 10 ml of BHIB and incubated at 37 °C for 24-48 hours. The broth culture was then streaked on the surface HiCrome™ Listeria Agar Base, with selective supplement, a selective and differential agar medium suggested for quick and direct identification of *Listeria* species, and then incubated at 36°C  $\pm$  2°C for 24-48 hours. Then isolates colonies were detected.

### *Listeria monocytogenes* Identification

The suspect colonies were identified as *L. monocytogenes* using a selective and differential agar (as mentioned previously), after which they were submitted to biochemical tests such as Gram staining, oxidase, and catalase tests, carbohydrate fermentation tests (mannitol, rhamnose, glucose). As pathogenic *Listeria* spp. was distinguished from non-pathogenic spp., all of the described *Listeria* isolates were tested for hemolysis on (5%) sheep blood agar (SBA). The *L. monocytogenes* isolates were also

confirmed using a Real-time PCR method to determine the presence of a particular virulence feature, hlyA gene.

**Molecular detection of *L. monocytogenes* hlyA gene:** **DNA extraction:** By using Genomic DNA Extraction Kit, Genome Quick Bacteria (Dongsheng Biotech Co., Korea). This kit provides a straightforward and quick approach for obtaining high-quality genomic DNA from Gram-Positive and Gram-Negative Bacteria, which may then be utilized as the analysis target.

#### Detection of *L. monocytogenes* hlyA gene

Quantitative Real-time PCR (Q-PCR) assay is the confirmation process to determine the

presence of *L. monocytogenes* hlyA gene in the clinical specimens that gave positive results in selective and differential HiCrome™ *Listeria* agar base and biochemical tests previously. The process was conducted by specific detection of hlyA gene in the Purified DNA. Positive results in detection hlyA gene observed as amplification exponential florescent curves (Presence of Taqman Probe) at specific computer screen attached with the thermal cycler system. The design of primers and probe for hlyA gene was conducted using Beacon Designer and used in this study are show in **Table (1)** as follows:

**Table 1. Primers of hlyA gene used in this study for species detection *Listeria monocytogenes* by Real-time PCR**

Primers		Sequence 5'-3'	Target gene	Fragment size (bp)
L.M	F	GGCTCCGCAAAGATGAA	Hly	105
	R	GGGAATCCTGGTGTTC		
L.M. (FAM-BHQ)		CGACGGTAACCTCGGAGACTTACGA	Hly	105

#### Real-time PCR reaction preparation

Amplification was carried out according to the following thermal and cycling conditions as shown in **Table (2)**.

**Table 2. Program of real-time PCR thermocycling conditions**

Steps	Temperature	Time	No. of cycle
Initial denaturation	95	900 sec	1
Denaturation	95	5 sec	40
Annealing	60	20 sec.Acquire to [Green]	
Extension	72	10 sec	

#### Q-PCR melting curve with SYBR Green

Moreover, to confirm our specimens that gave positive results in the previous step were highly pure, additionally there were no contaminations in our work or primer-dimer and other non-specific products, so melting

curves were done. Program conditions with a total volume of 20µl are mentioned in **Table 3** to detect hlyA gene of the same primers above with SYBR Green instead of the probe in this technique.

**Table 3. Program conditions to detect hlyA gene by Melting curve**

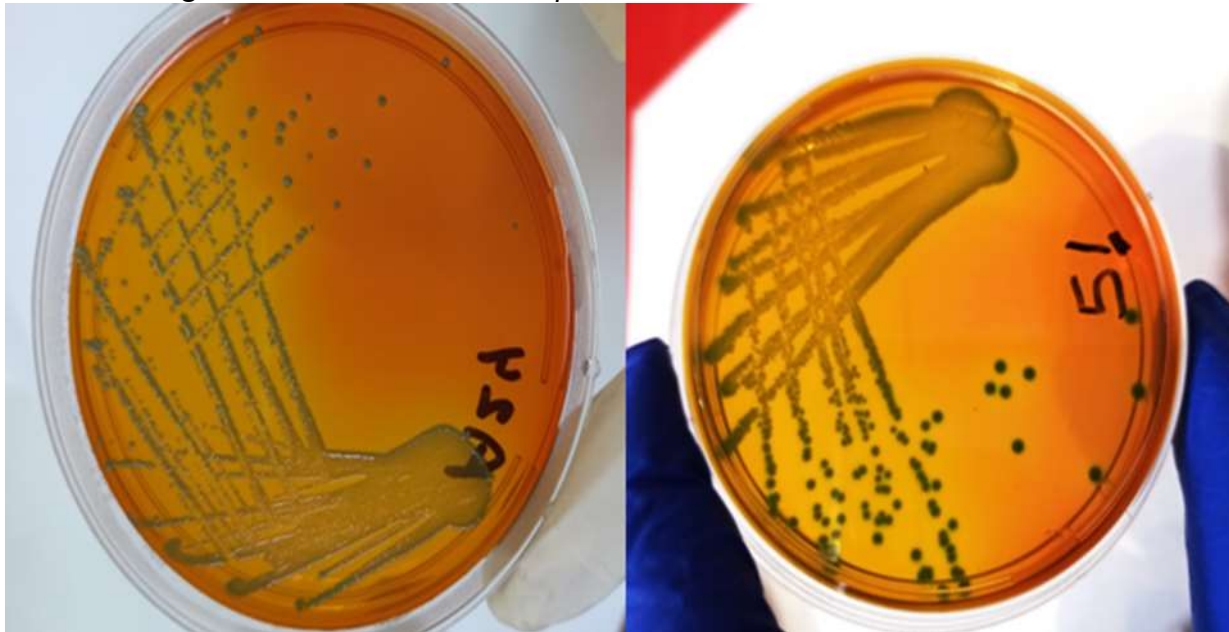
Steps	Temperature	Time	No. of cycle
Initial denaturation (Hold)	95	12 min	1Cycle
Denaturation	95	10 sec.	Cycling (5 repeats)
Annealing	58	20 sec.	
Extension	72	15 sec.	Cycling (40 repeats)
Denaturation	95	10 sec.	
Annealing	58	25 sec.	
Melting	(50-90 degrees)	Acquire to [Green]	Hold for 5 sec.
	Rising by 1.00 degrees	each step, Acquire to[Green]	

## RESULTS AND DISCUSSION

### Isolation and identification of *Listeria* spp

In this work, *Listeria* spp. were confirmed and identified using HiCrome™ *Listeria* Agar Base, Modified (HIMEDIA M1417). The selective chromogenic measurement of  $\beta$ -glucosidase activity, as well as rhamnose fermentation, is the bases of this medium. The pure chromogenic substrate in the medium is hydrolyzed by *Listeria* species, resulting in blue-colored colonies. Other organisms cannot use the chromogenic substrate because the  $\beta$ -

glucosidase activity is peculiar to *Listeria* spp., resulting in white colonies. As well as the feature of rhamnose fermentation is used to distinguish *Listeria* spp (19). *L. monocytogenes* colonies were bluish-green with a yellow halo (rhamnose positive) **Figure (1)**. HiCrome™ *Listeria* Selective Supplement (FD181) is included in this medium, which inhibits the development of most Gram-positive and Gram-negative bacteria, as well as yeasts and molds.



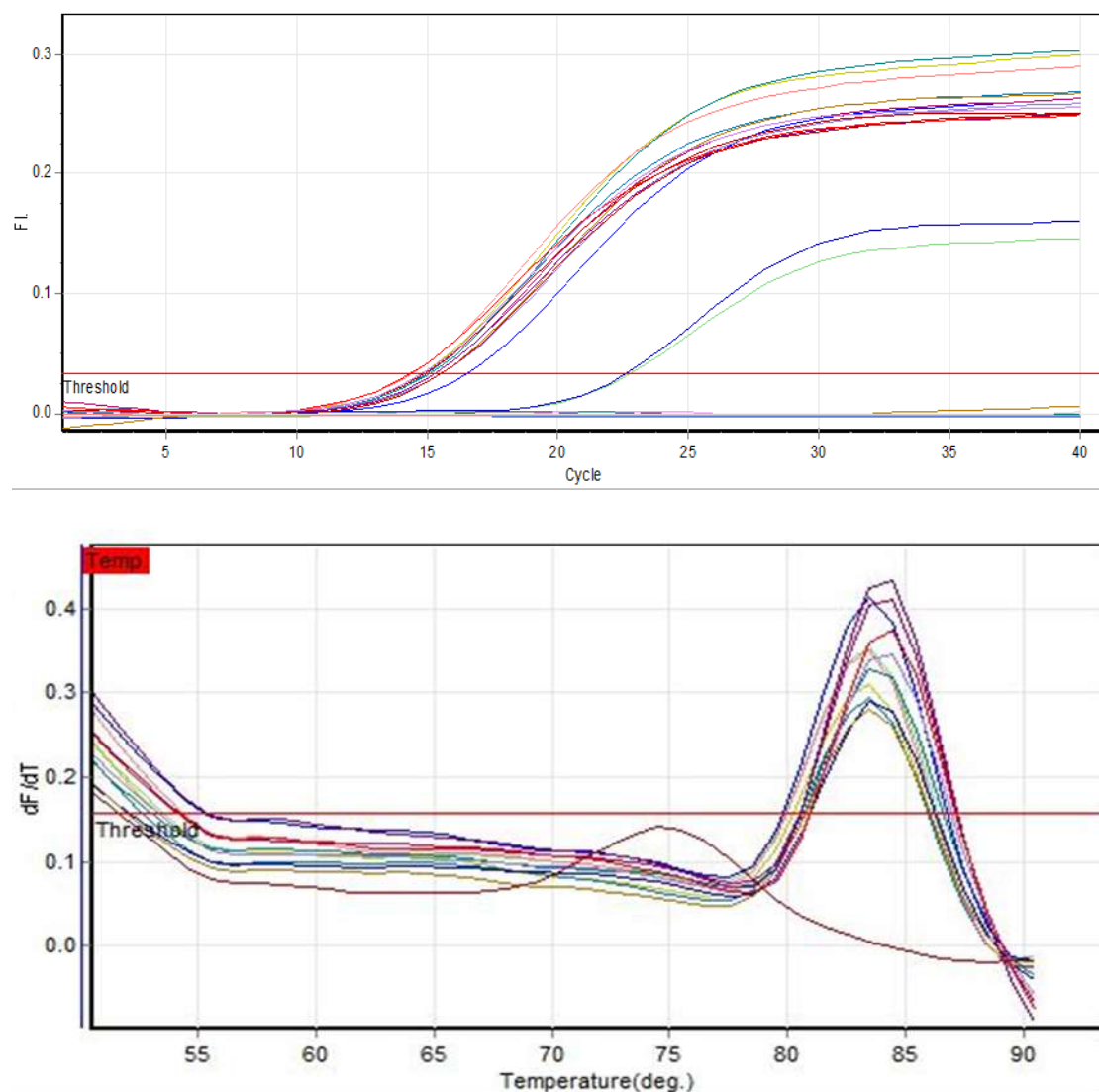
**Figure 1. *Listeria monocytogenes* colonies on HiCrome™ *Listeria* Agar Base selective and differential agar (colonies that are bluish-green with a yellow halo).**

The results of primary identification using biochemical tests were (Gram Staining (+ve), Catalase Positive (+ve), Oxidase Negative (-ve), carbohydrate fermentation D-mannitol Negative (-ve), rhamnose and D-glucose Positive (+ve), Hemolysis was  $\beta$ -hemolysis) and for HiCrome™ *Listeria* Agar Base revealed that out of the 200 (patients and control) specimens, from 176 miscarriages women analyzed, it was found that 117 (66.5%) specimens were Negative for *L. monocytogenes*, while 59 (33.5%) specimens from abortion cases were positive. Also, all 24 women control gives Negative to this bacterium. Abdalla *et al.* (1) identified *L.*

*monocytogenes* from aborted women by using biological and biochemical properties. In addition to molecular diagnosis, the microbes' isolation and identification were validated using staining, culture, morphological, and biochemical testing (2, 3).

**Identification of *L. monocytogenes* by Q-PCR:** The *hlyA* gene is one of the important virulence characteristics. Therefore it was chosen as the best target for the detection of this bacterium by real-time PCR. To find genetically common characteristics of newly isolated 57 (32.4%) positive specimens for *L. monocytogenes hlyA* gene was amplified by Q-PCR and mention in **Figure (2)**.





**Figure 2. Real-time PCR for detection hlyA gene of *Listeria monocytogenes*. (Quantitation Data (Green), *Listeria monocytogenes*, Green filter – FAM)**

The results of Q-PCR confirmed the previous results of HiCrome™ *Listeria* Agar and biochemical tests, where fifty-seven isolates from all positive women (n=59) showed the presence of the *hlyA* gene by Real-time PCR. Both molecular and traditional methods are effective in identifying *L. monocytogenes*, the molecular method remains the most accurate. For this small variation in results is attributed to the possibility of weak bacterial growth in these two samples, which resulted in DNA loss during the extraction process. The designed primers specifically amplify the *L. monocytogenes hlyA* gene, which encodes listeriolysin O and produces reproducible quantitative data over a large dynamic range of concentrations. The real-time PCR-*hly* assay demonstrated useful as a supplement to traditional bacteriological CSF culture by improving the speed and accuracy of

diagnosing *L. monocytogenes* CNS infection. Furthermore, the quantitative results supplied may be valuable for the follow-up of patients undergoing treatment in specific cases (10). On the other hand, (12) revealed that specificity of molecular detection of *hly* gene for *L. monocytogenes* was 100%, in comparison with other *Listeria* isolates, and under certain settings, this PCR detection method, which is relatively sensitive and more specific, could be used as a quick diagnostic method from contaminated food. Following amplification, a melt curve analysis was performed to verify the specificity of the amplified products by their specific melting temperatures  $T_m$  (6). The protein with pore-forming activity is the most important factor in vacuole lysis. The 58-kDa protein (Listeriolysin O) is encoded by the (*hlyA*) gene. The *hlyA* gene also has been targeted for

the development of PCR-based tests for *L. monocytogenes* detecting (9). According to the findings of a study conducted in Erbil, Iraq, the Q-PCR detection method, which included a set of primers and a hybridization probe, was very effective in monitoring the *L. monocytogenes hlyA* gene in meat (16). In one study in Italy, the Italian National Reference Laboratory for *L. monocytogenes* of Istituto Zooprofilattico Sperimentale dell' Abruzzo e del Molise (IZSAM) received a total of 37 clinical strains isolated from patients with listeriosis symptoms and 1374 strains correlated to the outbreak for outbreak investigation. A real-time PCR assay was discovered to be capable of screening thousands of *L. monocytogenes* strains in a short amount of time, saving time and money (17). The presence of virulence factors associated genes, *actA*, *hlyA*, *plcA*, and *prfA* in all strains with high antibiotic resistance indicated that *L. monocytogenes* strains were highly virulent, according to a research in Iraqi pregnant women at Al-Diwaniya hospitals, that included determination of virulence genes as well as antimicrobial susceptibility (4). *Listeria monocytogenes* can infect asymptomatic pregnant mothers, fetus infections are a dangerous illness that can lead to premature birth, abortion, sepsis, CNS involvement, or even death. If a pregnant woman exhibits symptoms similar to influenzas, such as headache, myalgia, fever, diarrhea, or any other digestive-related symptoms, the performance is practically identical to influenza (18).

## CONCLUSION

The prevalence of *L. monocytogenes* in cases of spontaneous abortions among Iraqi aborted women was 32.4%. It seems that the *hlyA* gene has an essential role in the early and accurate identification of the bacteria in aborted women by using Q-PCR. Additional studies are documented to investigate the routine monitoring of pregnant women for *L. monocytogenes* to reduce the illness load and manage treatment.

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