

# Detection of *hly A* gene in *Listeria monocytogenes* Isolated from Cerebral Spinal Fluid of Some Iraqi Meningitis Children

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## Abstract

*Listeria monocytogenes* is capable of causing serious invasive illness and may be responsible for several disorders, like acute meningitis, The this study aims to evaluate the reliability of molecular techniques and in particular, to evaluate the target *hlyA* gene as an alternative tool in order to identify *Listeria monocytogenes* meningitis. A lumbar puncture samples were obtained from 200 children patients from the period at January to August, 2016 admitted to Central Child Hospital (Al-Eskan) located in Baghdad province. In order to detect listeria infection, in conjunction with molecular detection of *hlyA* gene in *L. monocytogenes* isolates. *L. monocytogenes* was successfully detected and isolated from 10 (5%) patients. PCR technique was conducted to identify virulence associated genes(*hlyA* gene). All three isolates were found possessed flanking region sized 510bp of *hlyA* partial gene. We concluded that molecular diagnosis using Conventional PCR is fast and reliable diagnosis method contributes to shortening the time necessary to start specific treatment and monitoring the efficacy of therapy.

**Keywords:** *Listeria monocytogenes*, CSF, *hly A* gene, PCR

## INTRODUCTION:

*Listeria monocytogenes* is a Gram-positive short rod, beta-hemolytic, facultative intracellular pathogen in humans and animals; causing severe infection (listeriosis), with mortality rate range between of 25–30% of cases [1]. Mortality of listeria meningitis has remained high [2]. Listeriosis in both invasive and systemic infecting pregnant women and newborn leading to septicemia, sepsis, meningitis, encephalitis, and abortion, or non-invasive, appear in healthy individuals causing gastrointestinal and it is self-limiting [3]. Infection by *Listeria monocytogenes* is mediated by a number of virulence factors. The most important virulence factors is listeriolysin O (LLO), it is virulence associated gene important to cause infection and work as a pore- forming toxin [4], and it is found only in virulent strains of this bacteria. LLO virulence factor can serve in bacterial diagnosis as an indicator of the presence of *L. monocytogenes* in clinical and food samples [5]. *L. monocytogenes* has been confused with Streptococci, Pneumococci, diphtheroids, and *E. coli* in Gram film. In many laboratories, *L. monocytogenes* is incorrectly identified as diphtheroids and is discarded as a contaminant. Some researchers considered that if all diphtheroid - like bacteria showing beta- hemolysis are presumptively identified as *Listeria*. So, the researchers must use modern methods to diagnose Listeria like CHROMagar *Listeria*, molecular subtyping methods such as Multi Virulence-Locus Sequence Typing (MVLST), and Polymerase chain reaction (PCR) techniques [6 and 7]. PCR is conceded to be more reliable than conventional identification because it is based on stable genotypic characteristics rather than relying on biochemical or physiological traits, which can be genetically unstable. PCR is a sensitive and rapid technique to detect *L. monocytogenes* in the clinical sample [8]. PCR has high sensitivity and specificity for many infections of the CNS

and can be done with small volumes of C.S.F and it is faster and more sensitive than culture for bacterial meningitis. But PCR technique is expensive [9]. PCR has a sensitivity of (95-100) % for *L. monocytogenes* [10].

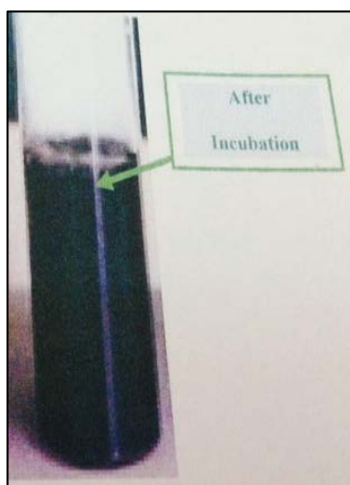
## MATERIALS AND METHODS:

Cerebrospinal fluid (CSF) samples were obtained from 200 children patients with acute meningitis admitted Central Child Hospital (Al-Eskan) in Baghdad province, from the period between Januarys to August 2016. Two to 3 mL of the specimen was collected by a physician through sterile lumbar puncture needle between the fourth and fifth lumbar vertebra to a depth of (4-5) cm and adequated in two sterile screw tubes. All samples were tested immediately to avoid cells lysis to determine the total white blood cells count (W.B.Cs.), glucose concentrations, and protein concentrations, microscopic and cultural examination. Subjected for detection and isolation of *L. monocytogenes* from CSF, samples were centrifuged at 3000 rpm for 3min, after centrifugation, all specimens of cerebrospinal fluid were cultured on blood agar base (Himedia) and enrichment agar base (Oxoid) [7]. Bacterial whole genomic DNA was extracted using ZR bacteria DNA MiniPrep extraction kit according to the manufacturing instruction (ZR bacteria DNA MiniPrep) according to manufacturer instructions. Specific primers set (*hly AF*: 5'- ATA CCA CGG AGA TGC AGT GAC -3') and (*hly AR*:5'- TCT TCT TGC ATTTTCCC TT CAC -3') were supplied by (Integrated DNA Technologies company (IDT), Canada) that targeting *hlyA* gene flanking region. PCR reaction was conducted in 25µl of a reaction mixture containing 2µl of DNA, 12.5 µl GoTaq0T® Green Master (Promega, CA), (0.5 µl) 25mM MgCl<sub>2</sub>, 2µl of (10 Pmol\ µl) from each primer, 2µl of DNase free water. PCR condition optimized several time through experimenting three gradient temperature (57°C, 58°C, 59°C) with

different time till reach to the optimal condition. Amplification program was 1 cycle at 94°C for 10 min; 35 cycles of Secondary denaturation at 94°C for 20 Sec, annealing at 57°C for 45 Sec, extension at 72°C for 1min; 1 Cycle final extension at 72°C for 10min, using the thermocycler (Eppendorf). The PCR product was subjected to 1.5% Agarose gel electrophoresis, stained with Red Safe dye and visualized under UV light (Imagemaster VDS, Pharmacia Biotech, USA) [11,12].

**RESULTS AND DISCUSSION:**

From 200 CSF samples tested in the present study, only 10 (5%) of samples were found positive to *L. monocytogenes* and successfully cultivated on culture media. Enrichment agar base was changed to black color which indicated the ability of *L. monocytogenes* to hydrolyze Aesculin that is shown in figure (1). The medium utilizes the selective inhibitory components such as lithium chloride, acriflavine, colistin, sulphate, cefotetan, or amphotericin B, fosfomicin, and cycloheximide which inhibit Gram-negative, and most Gram-positive bacteria, and the indicator system represented by Aesculin and ferrous iron as indicators for the isolation or differentiation of *L. monocytogenes*. When *L. monocytogenes* hydrolyses Aesculin, black zones were reproducing around the colonies because of the formation of black iron phenolic compounds that derived from the glucon. [13]. Biochemical CSF test indicated the appearance was cloudy and yellowish. The WBCs number was 7000 cell/ml; the concentration of protein was 63 gm/l and glucose concentration was 7.5mol/l. The normal CSF should be free from bacteria and its appearance is a clear colorless free of any pigmentation fluid, WBC ranging in adult 0-5/mm<sup>3</sup>, protein concentration Proteins: 20-40 mg/dL, and glucose concentration 45-80 mg/dL. Our finding of CSF parameter was indicated the bacterial infection in suspected patients which is agree with Pesco (2010) and (Herson *et al*., (2011) who refers to that different infection or medication can change the biochemical composition of CSF, which is helpful in the diagnosis of a wide range of disease effecting central nervous system.



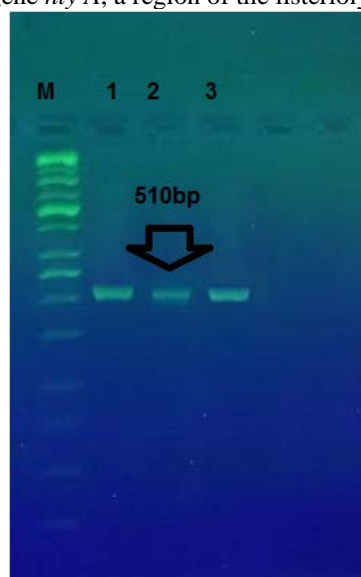
**Figure 1:** *L. monocytogenes* appearance in enriched based agar.

The extracted DNA result is shown in Figure (2). Genomic DNA bands were sharp with good quality on Agarose gel. DNA concentration is ranging between 54-294 ng/μL with perfect purity 1.6 - 1.9 which indicated that ZR bacteria DNA MiniPrep kit features was fast, easy processing, produced high quality and good purity of extracted DNA. It is suitable for DNA used in PCR technique because molecular biological studies of bacteria, such as the PCR techniques, require pure DNA [11]



**Figure (2):** Agarose gel electrophoresis of the total genomic DNA for *L. monocytogenes* isolated from CSF sample. Fragments were fractionated by electrophoresis on 2% agarose gel at 5 vol/cm for 1:15 hour, and visualized under U.V. light after staining with Red safe dye.

PCR assay was used to identification of *hly A* gene using specific primer in *L. monocytogenes*. All teen samples shown in figure (3) positive result for *hly A* gene represented by the appearance of single band sized 510bp without non-specific bands as mansion previously in [4 and 6]. The results of this study demonstrated that *hly A* gene primer based PCR method had high sensitivity and specificity in detecting *hly A* gene in *L. monocytogenes* isolated in the present study consistent with Jeyaletchumi.(2010)PCR subjected to detect the virulence associated gene *hly A*, a region of the listeriolysin O gene.



**Figure (3):** PCR product for *hlyA* gene electrophoresed on 1.5% Agarose gel, 3 Vol/cm, stained with Red Safe and visualized under U.V. light. Lane M: 100-bp DNA ladder; Lane 1, 2, 3 *hlyA* gene bands sized 510 bp.

Also agree with Dillon and Patel (2015) finding that an etiological diagnosis based on cultures, and serological identification could be difficult because numerous pathogens can cause encephalitis, therefore pathogen-specific PCR assays consider the excellent method for detection and identification. The current result considered the first molecular study based on *hly A* gene in Central Children Hospital /Al-Eskan / Baghdad province to detect *L. monocytogenes* in children with acute meningitis. PCR techniques may be preferred when some cells do not grow well in the selective media. In previous study conducted by Shareef *et al.*, (2008) in Al-Mosel city located north of Iraq was depend it on conventional method to detect *L. monocytogenes* showed that the rate of *L. monocytogenes* infection was 14%, bacterial infection associated with an increase in the WBC number 96 cell/ml<sup>3</sup> in glucose level in compared with the controll. Many researchers such as Vázquez-Boland *et al.*,(2001); Jaradat *et al.*, ( 2002); Furrer *et al.*, (2008), Trezey *et al.*, (2015); Low *et al.*, (2015) nominated and preferred using the *hlyA* gene in molecular detection of *L.monocytogenes* among many virulent genes. Shrinithiviahshini *et al* .,(2011) finding in his

study on the patients from south India PCR technique was used in combination with immunohistochemical technique the incidence considered especially in the elderly and immunocompromised patients. PCR technique has the rapid potential to revolutionize the ability to identify common, rare, or even newly identified pathogens from CSF samples, early detection of. Pesavento *et al.*, (2009) from Italy and Rahimi *et al.*, 2012 from Iran, indicated a high resistance of *Listeria spp.* to nalidixic acid, penicillin, tetracycline, and ciprofloxacin, and to a lesser extent to erythromycin, ampicillin, and chloramphenicol, also Thennings *et al.*, (2016) in refers to the antibiotic combination treatment including benzyl penicillin, ampicillin, and meropenem could be very effective in treating this bacteria. Invasive *L. monocytogenes* will facilitate effective antibiotic treatment since it is known to be resistant to the ordinary antibacterial meningitis treatment leads to a high mortality rate despite antibiotic treatment through randomized clinical trials so fast diagnosis and appropriate antibiotic response reduce the mortality rate among patients.

*Listeria monocytogenes* strain JP2008 listeriolysin O precursor (*hly*) gene, complete cds  
Sequence ID: KR185738.1.

| Alignment statistics for match #1 |        |   |           |           |     |
|-----------------------------------|--------|---|-----------|-----------|-----|
| Score                             | Expect | Identities  | Gaps      | Strand    |     |
| 893 bits(483)                     | 0.0    | 497/504(99%)  | 0/504(0%) | Plus/Plus |     |
| Query                             | 1      | GTATACCACGGAGATGCAGTGACAAATGTGCGGCCAAGAAAAGGTTATAAAGATGGAAAT  |           |           | 60  |
|                                   |        |   |           |           |     |
| Sbjct                             | 229    | GTATACCACGGAGATGCAGTGACAAATGTGCGGCCAAGAAAAGGTTATAAAGATGGAAAT  |           |           | 288 |
| Query                             | 61     | GAATATATCGTTGTGGGGAAAAAGAAGAAATCCATCAATCAAAATAATGCAGATATCCAA  |           |           | 120 |
|                                   |        |   |           |           |     |
| Sbjct                             | 289    | GAATATATCGTTGTGGAGAAAAAGAAGAAATCCATCAATCAAAATAATGCAGATATCCAA  |           |           | 348 |
| Query                             | 121    | GTTGTGATTGCAATTTTCGAGCCTAACATATCCAGGTGCTCTCGTGAAAGCGAATTCGGAA |           |           | 180 |
|                                   |        |   |           |           |     |
| Sbjct                             | 349    | GTTGTGAATGCAATTTTCGAGCCTAACATATCCAGGTGCTCTCGTGAAAGCGAATTCGGAA |           |           | 408 |
| Query                             | 181    | TTAGTAGAAAAATCAACCCGATGTTCTTCTGTCAAACGTGATTCAATTAACACTTAGAATT |           |           | 240 |
|                                   |        |   |           |           |     |
| Sbjct                             | 409    | TTAGTAGAAAAATCAACCCGATGTTCTTCTGTCAAACGTGATTCAATTAACACTTAGCATT |           |           | 468 |
| Query                             | 241    | GATTTGCCAGGAATGACTAATCAAGACAATAAAAATGTTGTAAAAAATGCTACTAAATCG  |           |           | 300 |
|                                   |        |   |           |           |     |
| Sbjct                             | 469    | GATTTGCCAGGAATGACTAATCAAGACAATAAAAATGTTGTAAAAAATGCTACTAAATCG  |           |           | 528 |
| Query                             | 301    | AACGTTAACACGCAGTAAATACATTAGTGAAAGATGGAATGAAAAATATGCTCAAGCT    |           |           | 360 |
|                                   |        |   |           |           |     |
| Sbjct                             | 529    | AACGTTAACACGCAGTAAATACATTAGTGAAAGATGGAATGAAAAATATGCTCAAGCT    |           |           | 588 |
| Query                             | 361    | TATCCAAATGTAAGTGAAAAAATGATTATGATGACGAAATGGCTTACAGTGAATCACAA   |           |           | 420 |
|                                   |        |   |           |           |     |
| Sbjct                             | 589    | TATCCAAATGTAAGTGCAAAAAATGATTATGATGACGAAATGGCTTACAGTGAATCACAA  |           |           | 648 |
| Query                             | 421    | TTAATTGCAAAATTTGTACGGCATTAAAGCTGTAAATAATAGCTTGAATGTAAACTTC    |           |           | 480 |
|                                   |        |   |           |           |     |
| Sbjct                             | 649    | TTAATTGCAAAATTTGGTACGGCATTAAAGCTGTAAATAATAGCTTGAATGTAAACTTC   |           |           | 708 |
| Query                             | 481    | GGCGCAATCAGTGAAGGGAAAATG                                      |           |           | 504 |
|                                   |        |   |           |           |     |
| Sbjct                             | 709    | GGCGCAATCAGTGAAGGGAAAATG                                      |           |           | 732 |

**Figure (4): Sequencing of *hly A* gene of *Listeria monocytogenes* obtained from Gene bank. Query represents of sample; Subject represent of database of National Center Biotechnology Information (NCBI).**

## Sequencing

The sequencing and BLAST analysis of partial *hly A* gene as seen in table (1). The results of the sequencing showed congruence with isolation *Listeria monocytogenes* isolated from cerebral spinal fluid of amplified product of *hly A* gene appeared 99% compatibility from 229 to 732 number of nucleotide of the gene bank, and have number score (893) bits, expect (0), and corresponds to the accession number ID: KR185738.1. The results as shown after alignment of product amplification of *hly A* gene having one Transition A305G (GAG>GGG), and having six Transversion C260G,( CCG>CGG)A356T(AAT>ATT),C465A(AGC>AGA),T503A(ATT>AAT),C605A (GCA>GAA) and G664T(GGT>TGT) from the Gene Bank. The *hly A* gene were registered after the correspondence of The National Center for Biotechnology Information and obtained an accession number and became a reference to Iraq and the Middle East and the world under number MH016280.

## CONCLUSION

This study improved the applicability of PCR diagnostic assay targeting *hlyA* gene as an alternative tool in order to identify *L. monocytogenes* meningitis in meningitis Iraqi patients, which has a significant impact on diagnosis approaches to CNS infectious diseases.

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