Chronic Diseases Journal

DOI: 10.22122/cdj.v11i3.835 Published by Vesnu Publications

Evaluation of hly-A and 16S rRNA genes for detection of Listeria monocytogenes in raw milk sample

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Short Communication

Abstract

BACKGROUND: Listeria monocytogenes (L. monocytogenes) is a pathogen that causes meningitis, septicemia, and abortion in humans. These microorganisms can be transmitted through food. The aim of this study is a comparison of the two important target hemolysin A (hly-A) and 16S ribosomal ribonucleic acid (16S rRNA) genes in the early detection of L. monocytogenes in raw milk samples by polymerase chain reaction (PCR) assay.

METHODS: In this cross-sectional study, one hundred samples of raw milk were collected from Damavand City, northern part of Iran, in 2015. After deoxyribonucleic acid (DNA) extraction, the PCR was optimized by using hly-A and 16S rRNA genes. After purification, the PCR product was cloned using a thermocontact TA cloning kit and vector pTZ57R. Data analysis was performed using MINITAB software (Mtb.exe). The chi-square test was used for the statistical test ($P < 0.05$).

RESULTS: Out of 100 samples, 13 samples have been positive for hly-A and 11 samples have been recognized as positive for 16S rRNA.

CONCLUSION: Results of our study show that the hly-A target gene has higher performance in molecular diagnosis of L. monocytogenes than 16S rRNA.

KEYWORDS: Listeria Monocytogenes; 16S rRNA; Hly-A

Date of submission: 20 Mar. 2022, ***Date of acceptance:*** 25 Dec. 2022

Citation: Bahramian E, Shahhosseiny MH, Shakib P, Zolfaghari MR, Ahmadi S. **Evaluation of hly-A and 16S rRNA genes for detection of Listeria monocytogenes in raw milk sample.** Chron Dis J 2023; 11(3).

Introduction

Listeria monocytogenesis (L. monocytogenes) can cause meningitis, encephalitis, and sepsis, especially in pregnant women, infants, and the elderly.[1,2] This microorganism is also transmitted through food.[3-5] The mortality rate of listeriosis has been reported to be 30% and in vulnerable people, up to 75%.[6] Isolation of L. monocytogenes from food by culture and its identification by biochemical methods takes 7-8 days. Currently, the most commonly used diagnostic method for L. monocytogenes is crop cultivation due to its time consumption, low efficiency (due to low bacterial count), and the problem of differential diagnosis of other bacteria. To overcome the limitations of traditional diagnostic testing, deoxyribonucleic acid (DNA)-based methods have been developed to detect various microbial pathogens.[7] For the detection of L.

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monocytogenes, several genes have been specifically used.[7-9] Many polymerase chain reaction (PCR)-based studies on L. monocytogenes have been based on determining the presence of pathogenic genes, including the hemolysin A (hly-A) gene.[10,11]

In our study, we have two genes including 16S ribosomal ribonucleic acid (16S rRNA) gene that contains consensus sequences, and the hly-A gene, which is a gene involved in the L. monocytogenes pathogenicity that produces listeriolysin O (LLO) (encoded by hly-A), which is used to detect L. monocytogenes. The hly-A gene is protected in all strains of L. monocytogenes.[12] Considering the importance of these two genes in the detection of L. monocytogenes, our main goal is to compare these two genetic markers in the molecular detection of L. monocytogenes in raw milk. Therefore, considering that it is very important to achieve a test that can accurately detect the presence of L. monocytogenes in any situation, the purpose of this study was optimized based on two sets of primers, one specific for Listeria (16S rRNA) and another for L. monocytogenes (hly) for detection of L. monocytogenes isolates.

Methods

Sampling: In this experimental study, in 2015, one hundred samples of raw milk were collected from different parts of Damavand City, Tehran Province, the northern part of Iran. Samples were transferred to the laboratory in cold conditions. DNA extraction from collected samples and standard strain into PTCC1163 was performed using DNG-Plus commercial kit (Sinaclon, Iran).

Optimization of the PCR method: To optimize the PCR technique, the appropriate concentration and volume of the components required for this method were evaluated, and finally, the required values were obtained in 25 µl PCR reaction that consisted of 5 µl of 5x master mix PCR, 0.5 µl deoxynucleotide triphosphate (dNTP), 2 µl magnesium chloride ($MgCl_2$), 0.5 µl Taq DNA polymerase enzyme, 0.5 µl of each 20 pM primer, 13 µl of distilled water [deuterium depleted water (DDW)], and 2 µl of DNA template. PCR amplification was carried out with thermal conditions: initial denaturation at 94 ◦C for 3 minutes, followed by 35 cycles each of 30-second denaturation at 94 ◦C, 30 seconds of annealing at 60 ◦C, 90 seconds of extension at 72 ◦C, and final extension at 72 ◦C for 5 minutes (Table 1).[13]

Table 1. Primers used for amplification of hemolysin A (*hly*-A) and 16S ribosomal ribonucleic acid (16S rRNA) genes in the present study

Genes	Primer sequences (5′ to 3′)
Hly-A	Forward: -CGCAACAAACTGAAGCAAAGG- Reverse: -TTGGCGGCATTTGTCAC-
16S rRNA	Forward: -TGTTAATGAACCTACAGGACCTTC- Reverse: -TAGTTCTACATCACCTGAGACAGA-

Hly-A: Hemolysin A; 16S rRNA: 16S ribosomal ribonucleic acid

PCR product cloning: After purification, the PCR product was cloned using thermocontact TA cloning kit and vector pTZ57R. The resulting plasmids were extracted. PCR-containing plasmids were then confirmed by PCR.

Determination of the sensitivity and specificity of the PCR test: For this purpose, DNA was extracted from the standard strain of L. monocytogenes, whose concentration was measured by a NanoDrop device. The DNA was prepared with dilution-specific concentrations. The DNA of Mycobacterium tuberculosis, Staphylococcus aureus, Legionella pneumophila, Lactobacillus acidophilus, Mycoplasma spp., Salmonella spp., Pseudomonas aeruginosa and Bifidobacterium bifidum, as well as positive and negative control samples were used to confirm the specificity.

Statistical analysis: Data analysis was performed using MINITAB version 14.2

(Mtb.exe). Considering the assumed prevalence of *hly* about P = 50%, test power of 80%, and test accuracy of d = 0.2, the sample size was estimated to be at least 96 using the following formula:

$$N = Z^2 * P * (1-P)/d2.$$

According to the selection of 100 samples (the most stringent number of samples in the statistical tests), our samples represent a normal society, which will be verified by testing the difference between the average of the two normal societies. Mean and standard deviation (SD) were used to describe quantitative data, frequency and percentage were used to describe qualitative data, and the chi-square test was used for the statistical test at the level of P < 0.05. (Project code: 15430507941002).

Results

In this study, over one year (2015), 100 samples (the most stringent number of samples in statistical tests) represented a normal population and we proved our hypothesis by testing the difference between the mean of two normal populations. 100 samples of raw milk were collected from livestock farms, and their DNA was extracted by the DNG-plus method and tested by PCR. 11 specimens were positive for the 16S rRNA gene (Figure 1) and 13 cases for the hly-A gene (Figure 2).

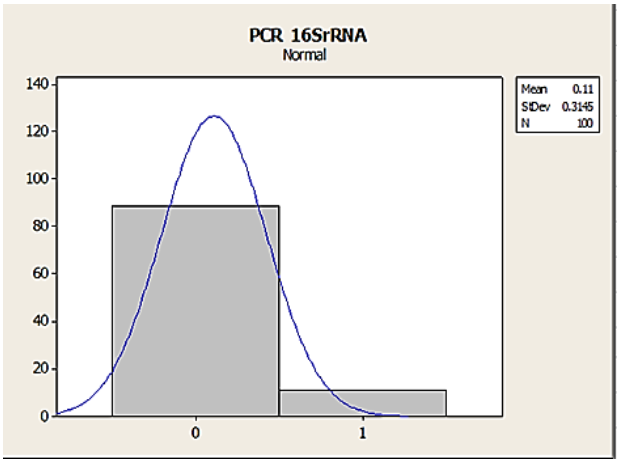

Figure 1. Normal distribution of the 16S ribosomal ribonucleic acid (16S rRNA) gene

Determination of PCR sensitivity and specificity: The PCR sensitivity test was performed by providing sequential dilutions of L. monocytogenes DNA using primers of both 16S rRNA and hly-A genes. The results of PCR assay sensitivity showed that with 100 copies of DNA, replication was performed and in titers, Less than 100 copies of a DNA band were not observed, which indicated the high sensitivity of the test. The results also showed that the optimized PCR test had very high specificity, so that it reacted only with the DNA of L. monocytogenes, and no reaction was performed with the DNA of other microorganisms.

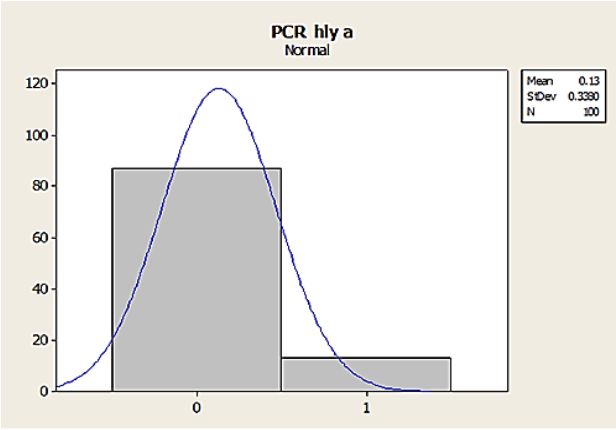

Figure 2. Normal distribution of the hemolysin A (hly-A) gene

Cloning the PCR product as a positive control using the 16S rRNA and hly-A: Twenty-eight hours after the transformation, white and blue clones appeared on the luria broth (LB)-agar plate containing X-gal, isopropyl β-D-1-thiogalactopyranoside (IPTG), and ampicillin antibiotics. Given the cloning principles in the vector pTZ57R/T, it was therefore expected that the white clones contained a recombinant plasmid. Blue clones were not recombinant and infected. After extracting the plasmid from white clones, a PCR reaction was performed using extracted plasmids according to optimized conditions. The product of the PCR

reaction was investigated using electrophoresis on the agarose gel. The observation of 226 bp and 210 bp proliferation in the PCR reaction on agarose gel confirmed the presence of recombinant plasmid in all selected clones. If we compile the first population with the results of the PCR test of the 16S rRNA gene and compile the second population with the results of the PCR test of the hly-A gene, the results of the expression of the superiority of the hly-A gene in 100 milk samples will be investigated. The test of the hypothesis will prove our theory as follows.

The test of the hypothesis proved our theory as follows:

1. The test hypotheses was formed.
2. The significant level was set at 0.05.
3. Null hypothesis was rejected when the value of the test index was greater than the value of Z in the table (Table 2).

Table 2. Test of the hypothesis in this research

	Statistical results
X1	0.11
X2	0.13
Variance 1	0.09
Variance 2	0.11
SD 1	0.31
SD 2	0.33
QH	4.33
Z(0.975)	1.96

SD: Standard deviation; QH: Queensland Health

Discussion

The main goal of this project was to reduce potential errors in the detection and separation of L. monocytogenes from various samples. Considering this goal, we used two sets of primers including one targeted against genus-specific 16S rRNA and another targeted against hly virulence genes of extracted DNA from L. monocytogenes in raw milk samples. The results of this study indicate that the hly-A gene target has the potential to detect more L. monocytogenes than the 16S rRNA gene. Razei et al. used hly-A gene and 210 bp primer pair for rapid detection of L. monocytogenes and announced the speed of testing for a rapid diagnosis of L. monocytogenes as 1.5 hours,[14] which is consistent with the current study. For this reason, determining the rows of 16S rRNA gene blocks is used as a standard method for identifying species, sex, and familial bacteria. Recently, organic bands have been used only in a small portion of the 16S rRNA gene to detect bacteria in the genus or higher levels.[3] Feng et al. detected L. monocytogenes by combining aptamer magnetic capture and loop-mediated isothermal amplification (AMC-LAMP). In their study, accuracy of AMC-LAMP was 100% and the result showed that AMC-LAMP was a useful assay for rapid detection of L. monocytogenes in food.[5] Zhan et al. used enzyme-linked aptamer assay (ELAA) with rolling circle amplification (ELARCA) test for the colorimetric revealing of L. monocytogenes and described the ELARCA method as sensitive, highly specific, fast, and low-cost for detection of L. monocytogenes.[6] Ye et al. detected L. monocytogenes in vegetables using the 16S rRNA gene, which did not show a statistically significant difference between this method and the culture method. But molecular method was faster than the culture method.[7]

This result indicates that molecular testing is more sensitive than routine culture. In this investigation, according to our purpose, we finally optimized PCRs based on two sets of primers, one specific for Listeria (16S rRNA) and another for L. monocytogenes (hly) for rapid detection of L. monocytogenes in raw milk samples. One of the limitations of this study is the lack of samples due to time, and the lack of sufficient funds to investigate other new molecular techniques for rapid diagnosis of L. monocytogenes.

Conclusion

Primer hly-A used in this study is better than the 16S rRNA gene. It is a primer for the detection of L. monocytogenes. Besides,

according to the calculations done using the MINITAB software, the test statistic is greater than Z (0.975), which rejects the assumption of the equality of the two hly-A and 16S rRNA genes, The results of this research showed that the hly-A gene was more accurate than the 16SrRNA gene in identifying L. monocytogenes.

Conflict of Interests

Authors have no conflict of interests.

Acknowledgments

We would like to thank directors at the Islamic Azad University, Qom Branch, Qom, Iran, for their assistance and collaboration.

Financials support and sponsorship

This study was not financially supported.

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