Listeria monocytogenes Specific Detection Employing Novel Primers with In Silico Study to Detect Listeriolysin O Protein-Coding Gene *hly*

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Abstract. *Listeria monocytogenes* is a pathogenic bacterium, which prevalence in the aquatic ecosystem and seafood products become a major threat to public health. Several virulence genes had been targeted for the PCR (polymerase chain reaction) detection of *L.monocytogenes* including hly coding for listeriolysin O precursor, which resulted in many reported primers. This paper presents newly paired primers for selective PCR detection of *L.monocytogenes* strains based on hly sequence by in silico approach using Primer3Plus and In Silico Programs. The quality of the newly paired primers was compared to the previously reported ones from various studies using Autodimer program. As result, two paired primers HlyOF: 5'-GACAAATGTGCCGCCAAGAA-3' and ELMHLYR: 5'-GCGCTTGCAACTGCTCTTTA-3, as well as lmo0202_q_F: 5'-CTCCGCCTGCAAGTCCTAAG-3' and HlyR: 5'-CTTCACTGATTGCGCCGAAG-3' were found to be the best in performance in virtual PCR compared to other previously reported pairs of primers. Using these primers, positive 596-bp and 594-bp PCR products, respectively, could be amplified from each of all genome sequences of *L.monocytogenes* strains (41 strains) available in the database of In Silico Program, but not from those of other Listeria species. Compared to previously reported primers, the newly paired primers have better theoretical quality based on Tm difference. The F: 5'-GACAAATGTGCCGCCAAGAA-3' primer is a novelty as it has not been reported anywhere and named as HlyO. As a conclusion, this in silico study confirmed that hly gene is an apparent marker gene, which only present in *L.monocytogenes*, but absent in other Listeria species. Hence, the newly paired primers are the potential to be used as a selective PCR detection component of *L.monocytogenes* of various strains.

Keywords: Systemic Lupus Erythematosus (SLE), Hemoglobin Level, Leukocyte Count, ESR.

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1. Introduction

Seafood is an important global food commodity playing a vital role in healthy diet. On the other hand, one of the four food groups most likely to contribute significantly to global outbreaks and high rates of foodborne illness is seafood. Listeria is one of the main human infections that cause foodborne deaths in a number of nations [1][2].

The FDA reported that Listeria bacterial genus contains six species: *Listeria monocytogenes, L. innocua, L. seeligeri, L. welshimeri, L. ivanovii*, and *L. grayi*. Among these species, *L. monocytogenes* and *L. ivanovii* and are pathogens for mice and other animals. Yet, only *L. monocytogenes* is pathogenic to humans for causing listeriosis leading to foodborne outbreaks. Listeriosis disease has a mortality rate of 20–30 % among its vulnerable groups. The groups consist of people with weakened immune systems, adults between the ages of 65 and 75, pregnant women, fetuses, and newborns [3].

Numerous nations have published extensive reports on the prevalence of *L. monocytogenes* in aquatic food products and related ecosystems [4][5]. The food products that include L. *monocytogenes* most frequently are frozen RTE (ready-to-eat) seafood products, such as smoked salmon and semi-preserved fish. This bacteria may cause foodborne illnesses when raw or undercooked seafood is consumed. [6].

L. monocytogenes generally infiltrate the food production chain through cros-contamination in production plants [3]. The virulence bacterium can survive in harsh conditions and form biofilms on a variety of surfaces [7]. These facts show the danger and insidiousness of *L. monocytogenes* make it a significant threat to the food industry for posing risk to human health [4][8].

PCR (polymerase chain reaction) detection is the initial critical step to control and prevent *L.monocytogenes* infection. Rapid detection of the causal pathogenic bacterium is essential for the early diagnosis of bacterial infection leading to the best therapy [9][10]. PCR (Polymerase Chain reaction)-based methods involving the amplification of a targeted gene fragment of *L. monocytogenes* such as HlyA-, PrfA, Iap, and SsrA using specific primers followed by sequencing the amplified PCR products are commonly used to detect *L. monocytogenes* [6][3].

Many studies have reported the possibility of hly as a DNA marker for *L. monocytogenes* bacterium [8][11][12][13][14]. To confirm this, however, an extensive investigation involving a large number of genomic DNA sequences of *L. monocytogenes* strains should be carried out. For example, a virulence factor of phospholipase called PI-PLC encoded by PlcA is considered as an important marker for Listeria species as it is expressed only by *L. monocytogenes* and *L. ivanovii*. However, the marker could only discriminate between the pathogenic and nonpathogenic groups of *Listeria* species [13][15].

Primers are critical components since they are the major determinants of sensitivity, specificity, and robustness of the method [16]. Oligonucleotide primers have been widely used in various PCR techniques, which specifically bind to the targeted DNA sequence on the template strand allowing the amplification of DNA sequence flanked by the primers. Optimal primer design is a critical parameter for a successful PCR [17][18]. Primer3 has been a popular and powerful primer design program extensively used in the past decades. Primer3Plus is the enhanced alternative for Primer3 available at http://www.bioinformatics.nl/primer3plus with a more powerful interface. In Primer3Plus, the default parameter settings are adequate for most purposes [19].

Designing good quality primers is important to support the successful outcome of a PCR experiment. Generally, good primers could avoid non-specific PCR products or amplicons appearing as varying sizes of bands or smears of bands on agarose gels. A poorly designed primer, even when all the other parameters are well-optimized, could lead to little or no product, due to nonspecific amplification causing reaction failure [17]. To avoid low-quality primers, after new primer sequences are designed, both forward and reverse ones should be screened for the possibility of primer-dimer and intramolecular hairpin formation. Such formation will lead to non-specific PCR products or inefficient amplification. Autodimer is among powerful primer quality checking program, which could perform primer-primer inter-comparison while based on conventional Watson-Crick base pairing rules evaluating interactions [20].

Today, whole-genome sequences of many *L. monocytogenes* strains have been reported from all over the world and available for access in Genbank database. Conducting "virtual" PCR with the bacterial genome sequences using specific primers is possible using in silico PCR tools freely available in http://insilico.ehu.es [21][22][23]. The specific Primers could be designed from also from a freely-available source such as Primer3Plus [19][23][24]. Results of such investigation could lead to information on whether the specific primers could selectively and simultaneously detect a large number of strains of a bacterial species from various origin worldwide. Besides, the results may confirm if the targeted gene is a biomarker for a specific species [23].

This paper reports a newly-designed specific primer from hly gene sequence of *L. monocytogenes* targeting the set of genome sequences of all strains stored in the database as templates in an in silico PCR. The investigation is also aimed to confirm if hly is the biomarker of the virulence bacterium using in silico approach. As a comparison, in silico investigation using the currently available primers designed from hly gene *L. monocytogenes* of in the last decades is also presented to check their quality.

2. Methods

The initial step of this work is a literature study conducted to get any information about the most distinct phenotypic characteristics of *L. monocytogenes*, especially related to the virulence of the bacterium. Next, responsible genes for the traits were targeted for primer design. The genes were then retrieved from The National Center for Biotechnology Information or NCBI database at https://www.ncbi.nlm.nih.gov. Specific primers were subsequently designed using Primer3Plus web-based tool freely available at https://primer3plus.com/cgi-bin/dev/primer3plus.cgi using the open reading frame (ORF) of the targeted gene sequences as input [19]. The settings of Primer3Plus in this work are just set by default.

In this study, the ability of *L. monocytogene* to produce specific toxin listeriolysin O is found to be one of the most distinct phenotypic traits of the microbe. The trait makes the gene coding for listeriolysin O precursor (hly) theoretically a potential biomarker candidate for the bacterium. Therefore, the open reading frame (ORF) of hly gene was targeted in the primer design step of this work. Quality of the designed primers was checked using Autodimer program available at https://www-s.nist.gov/dnaAnalysis/ [20]. Several designed primers obtained using Primer3Plus with hly gene sequence as input was then tested using in silico PCR at http://insilico.ehu.es/PCR/ on 43 Listeria genomes (applied to all species) available in the program database [21][22].

Subsequently, a post-in silico PCR analysis was carried out using all designed primers to make sure if the in silico PCR products or amplicons were truly part of hly genes and specific and selective only for *L. monocytogene*. The results could demonstrate if the primers have the potential to be used as a component of in vitro PCR for *L. monocytogenes* detection and if the amplified gene (hly) is the DNA biomarker of the of the species.

3. Result

From the literature study, it was found that *L. monocytogenes* can secrete a distinct toxin listeriolysin O, which precursor is uniquely coded by hly gene. The 3D structure model from hly sequence is represented by SWISS-MODEL. This model is to provide information on whether the hly sequence is truly a coding gene for the functional protein of listeriolysin O precursor of *L. monocytogenes*. Primer design in this study was then carried out based on hly gene Figure 1

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>M24199.1 L.monocytogenes listeriolysin O (hlyA) gene, complete cds
GAATTCTTCTGCTTGAGGGTTCATGTCTCATCCCCCAATCGTTTTTTATCGCCCTTTTTTAAAATACCCT
AAAAACATTAGGCAGTAACAACAATTGTTAGCTGTTGAAAGAAAGTCACGCTAAATGATGTTTTTTACAT
ATAGGATTTTATTATACAAATTTTGATTCGCAAAAGAAATGCATACATATTTAAAAACGGATTTATTTAG
ATGTTAAAATTGAAATAGAGTTAGTATATGGTTCCGAGGTTGCTCGGAGATATACTAACCCTTTTTTGTA
GGAATAATATATGTTAGTTGAATTTATTGTTTTTTATGATGTTTTTAATTGTTTGTTTTTCGGGGAAGTC
CATGATTAGTATGCCTAATCCTCGAACTTTTTCCGATGTTAAGTTGAGTACGAATTGCTCTACTTTGTTG
TTTAATGCTGCAGCATACTGACGAGGTGTGAATGTTAATGAAGTGGCGCTAATATGGTTAAGAAAAAGTT
TATTGTCCGCTTTGGAAGCTTGATAAGCAGTCTGGACAATCTCTTTGAATTTTGTTTTCTCACTCGGACC
ATTGTAGTCATCTTGAATTACTTGGTTAGGTGCGCCGAACTGCATGCCGAATTTGCGTGAGTTAATGACT
AATGGCTTTTTTGTGTGGTTCTCTGAAAGTAATAATATTTTTCCGCGGACATCTTTTAATGTAGGGATTT
TATTGCTCGTGTCAGTTCTGGGAGTAGTGTAAAAATAATCTTTATAAATGTTGATTAGTGGTTGGATCCG
ATAATCAAAACTATCGTTGCTGTTTTGCTCGTCTTTTAAACGCATAATAATGGTTTCTTTTGGATTTTTC
TTTAAAAATTGAGTAATCGTTTCTAATACACCTGAAAGTGATGCATTTAAAAAAATTGGCCCATGGTAAA
TGTTGAGATTGTCTTTTGCTCTAATATCGATGTACCGTATTCCTGCTTCTAGTTGTTGGTACAATGACAT
CGTTTGTGTTTGAGCTAGTGGTTTGGTTAATGTCCATGTTATGTCTCCGTTATAGCTCATCGTATCATGT
GTACCTGGTATAGAGAGCGCTGCTAGGTTTGTTGTGTCAGGTAGAGCGGACATCCATTGTTTTGTAGTTA
CAGAGTTCTTTATTGGCTTATTCCAGTTATTAAGCGAATATGCTTTTCCGCCTAATGGGAAAGTAAAAAA
GTATAAAATAAAACAGAGTAATAAAACTAATGTGCGTTGCAAATAATTCTTATACAAAATGGCCCCCTCC
TTTGATTAGTATATTCCTATCTTAAAGTGACTTTTATGTTGAGGCATTAACATTTGTTAACGACGATAAA
GGGACAGCAGGACTAGAATAAAGCTATAAAGCAAGCATATAATATTGCGTTTCATCTTTAGAAGCGAATT
TCGCCAATATTATAATTATCAAAAGAGAGGGGTGGCAAACGGTATTTGGCATTATTAGGTTAAAAAATGT
AGAAGGAGAGTGAAACCCATGAAAAAAATAATGCTAGTTTTTATTACACTTATATTAGTTAGTCTACCAA
TTGCGCAACAAACTGAAGCAAAGGATGCATCTGCATTCAATAAAGAAAATTCAATTTCATCCATGGCACC
ACCAGCATCTCCGCCTGCAAGTCCTAAGACGCCAATCGAAAAGAAACACGCGGATGAAATCGATAAGTAT
ATACAAGGATTGGATTACAATAAAAACAATGTATTAGTATACCACGGAGATGCAGTGACAAATGTGCCGC
CAAGAAAAGGTTACAAAGATGGAAATGAATATATTGTTGTGGAGAAAAAGAAGAAATCCATCAATCAAAA
TAATGCAGACATTCAAGTTGTGAATGCAATTTCGAGCCTAACCTATCCAGGTGCTCTCGTAAAAGCGAAT
TCGGAATTAGTAGAAAATCAACCAGATGTTCTCCCTGTAAAACGTGATTCATTAACACTCAGCATTGATT
TGCCAGGTATGACTAATCAAGACAATAAAATCGTTGTAAAAAATGCCACTAAATCAAACGTTAACAACGC
AGTAAATACATTAGTGGAAAGATGGAATGAAAAATATGCTCAAGCTTATCCAAATGTAAGTGCAAAAATT
GATTATGATGACGAAATGGCTTACAGTGAATCACAATTAATTGCGAAATTTGGTACAGCATTTAAAGCTG
TAAATAATAGCTTGAATGTAAACTTCGGCGCAATCAGTGAAGGGAAAATGCAAGAAGAAGTCATTAGTTT
TAAACAAATTTACTATAACGTGAATGTTAATGAACCTACAAGACCTTCCAGATTTTTCGGCAAAGCTGTT
ACTAAAGAGCAGTTGCAAGCGCTTGGAGTGAATGCAGAAAATCCTCCTGCATATATCTCAAGTGTGGCGT
ATGGCCGTCAAGTTTATTTGAAATTATCAACTAATTCCCATAGTACTAAAGTAAAAGCTGCTTTTGATGC
TGCCGTAAGCGGAAAATCTGTCTCAGGTGATGTAGAACTAACAAATATCATCAAAAATTCTTCCTTCAAA
GCCGTAATTTACGGAGGTTCCGCAAAAGATGAAGTTCAAATCATCGACGGCAACCTCGGAGACTTACGCG
ATATTTTGAAAAAAGGCGCTACTTTTAATCGAGAAACACCAGGAGTTCCCATTGCTTATACAACAAACTT
CCTAAAAGACAATGAATTAGCTGTTATTAAAAACAACTCAGAATATATTGAAACAACTTCAAAAGCTTAT
ACAGATGGAAAAATTAACATCGATCACTCTGGAGGATACGTTGCTCAATTCAACATTTCTTGGGATGAAG
TAAATTATGATCCTGAAGGTAACGAAATTGTTCAACATAAAAACTGGAGCGAAAACAATAAAAGCAAGCT
AGCTCATTTCACATCGTCCATCTATTTGCCAGGTAACGCGAGAAATATTAATGTTTACGCTAAAGAATGC
ACTGGTTTAGCTTGGGAATGGTGGAGAACGGTAATTGATGACCGGAACTTACCACTTGTGAAAAATAGAA
ATATCTCCATCTGGGGCACCACGCTTTATCCGAAATATAGTAATAAAGTAGATAATCCAATCGAATAATT
GTAAAAGTAATAAAAAATTAAGAATAAAACCGCTTAACACACACGAAAAAATAAGCTTGTTTTGCACTCT
TCGTAAATTATTTTGTGAAGAATGTAGAAACAGGCTTATTTTTTAATTTTTTTAGAAGAATTAACAAATG
TAAAAGAATATCTGACTGTTTATCCATATAATATAAGCATATCCCAAAGTTTAAGCCACCTATAGTTTCT
ACTGCAAAACGTATAATTTAGTTCCCACATATACTAAAAAACGTGTCCTTAACTCTCTCTGTCAGATTAG
TTGTAGGTGGCTTAAACTTAGTTTTACGAATTAAAAAGGAGCGGTGAAATGAAAAGTAAACTTATTTGTA
TCATCATGGTAATAGCTTTTCAGGCTCATTTCACTATGACGGTAAAAGCAGATTCTGTCGGGGAAGAAAA
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Fig. 1. Hly gene sequence

4. Discussion

In this study, we combined the use of Primer3Plus, Autodimer, and in silico PCR web-based tools for primer design and virtual PCR, to facilitate detection of foodborne pathogen *L. monocytogenes* based on its biomarker candidate gene [19][20][23].

Among the L. monocytegenes virulence factors is the secreted pore-forming toxin α-Hemolysin, listeriolysin O (LLO) encoded by hly. The toxin disrupts the phagosome allowing the proliferation of the bacterium in the cytosol [25].

Genome sequences of 5 of 6 members of Listeria genus reported by the FDA are available in the database of In Silico Program unless those of *L. grayi* species. This brings some limitation to this in silico study for inability to demonstrate if *hly* is truly absent in *L. grayi*

or if it is exclusively possessed only by *L. monocytogenes*. However, since there has been no report to date about the presence of hly in *L. grayi*, such restriction might be amended. ßTm of the primers is the temperature at which half the double-stranded DNA of primers are in the single-stranded random-coil state and half are in the double helical state. Tm is positively correlated with GC content and length of primer. Common parameters of primer design include 18–24 bp as optimal length of primers. It is advised to identify primers with minimum lengths and melting temperatures (T m) in the range of 59 to 68°C, with an ideal Tm of 63–64°C, in order to optimize for Real-Time PCR. Additionally, the Forward and Reverse primer pairs' Tms must to be within one degree Celsius of one another. [26].

Mohamed et al. (2016) found that based on hlyA gene sequence, the phylogenetic tree discriminates between the *L. monocytogenes, L. ivanovii, L. seeligeri*, and *L. innocua*. To screen for spontaneous virulence-attenuating mutations in *L. monocytogenes*. Maury et al., 2017 had reported the use of the hemolytic activity of LLO as a phenotypic marker. In L. monocytigenes, listeriolysin O (LLO) is a pore-forming toxin, a key virulence factor responsible for the ß-hemolytic phenotype of the bacterium. The hemolytic activity related to LLO is important for the detection and identification of *L. monocytogenes* in clinical and food microbiology because the phenotype characteristic is considered a cardinal marker for the pathogenic bacterium [27].

In the first newly paired primers, Sequence R: 5'-GCGCTTGCAACTGCTCTTTA-3, ELMHLYR or hlyr reported by Klein & Juneja in 1997 [28]. In the second newly paired primers, the sequence F: 5'-CTCCGCCTGCAAGTCCTAAG-3' is the forward primer sequence lmo0202_q_F [29], and hlyR: 5'-CTTCACTGATTGCGCCGAAG-3'. 5'-GACAAATGTGCCGCCAAGAA-3' primer is newly designed in this study and it has not been reported anywhere, so it is named HlyOF, a forward primer for hly of L. monocytgenes detection. Although Hly is different from the virulence gene homologs in other Listeria species, such L. seeligeri, it is conserved among isolates of *L. monocytogenes*. [30].

5. Conclusion

This in silico study confirmed that hly gene is an apparent marker gene, which only present in *L. monocytogenes*, but absent in other Listeria species. Hence, the newly paired primers are the potential to be used as a selective PCR detection component of *L. monocytogenes* of various strains.

Authors' Contributions. All authors contributed equally to this work.

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