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Discovery of *Listeria monocytogenes* High Prevalence in Pregnant Women who referred to the Tehran Women Reference Hospital by Real Time PCR Method

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Abstract

Background: Listeria monocytogenes is a gram positive and facultative anaerobic bacteria and Listeria species have a high spread in environment. Listeria monocytogenes is agent of meningitides, encephalitis and septicemia and could accrue during pregnancy. Detection of Listeria via culture method is difficult. The aim of this study was accessing of Listeria monocytogenes in pregnant women who refer to women hospital of Tehran via culture and Real Time PCR methods.

Methods: Two hundred cervix swabs samples were collected from pregnant women in seventh and eights mounts of pregnancy and transfer to laboratory in 2ml transport medium. 1ml was used for DNA extracting directly and other keep in refrigerator for cold enrichment among 4 weeks and then cultured. Primers were designed for amplifying *hly* gene which coded listeriolysin O.

Results: The result of culture and real time PCR methods were showed of 200 samples, 28 (14%) and 41(20.5%) were positive, respectively.

Conclusion: All of 13 false negative via culture, were positive in PCR technique. So, molecular method was more sensitive and recommended for applying. Moreover, the results showed high prevalence of carrier women in this time. Therefore, public screening was recommended for pregnant women.

Key words: Listeria monocytogenes, culture, molecular detection

INTRODUCTION

Listeria are small Gram-positive bacilli, ubiquitous, nonspore forming, facultative anaerobic bacteria that grow between −2 and 50 °C, with optimal growth between 30 and 37 °C [1]. The Listeria genus officially includes six species: Listeria monocytogenes, Listeria ivanovii, Listeria innocua, Listeria seeligeri, Listeria welshimeri, Listeria grayi [2]. Among those, L. monocytogenes is the most reported as pathogenic for humans (listeriosis) [3]. Human listeriosis outbreaks are most often associated with ready-to-eat food products that are consumed without prior cooking. Ingestion of foods contaminated with *L. monocytogenes* can result in listeriosis, a severe infectious disease characterized by meningoencephalitis, abortion, septicemia, and a high fatality rate (30%). Listeriosis predominantly affects certain risk groups, including pregnant women, newborns, elderly people and immunocompromised patients [4].

Classical microbiological methods for detection of Listeria involve the use of pre-enrichment and/or specific enrichment, followed by the isolation of bacteria in solid media and a final confirmation by molecular and/or serological tests. However, these procedures are not always effective and are extremely labour, intensive, requiring long time to yield a result. During the past decades, various alternative methods were developed to overcome these disadvantages, and a number of molecular methods were devised to reveal the presence of undesirable living microorganisms [5]. They are classical PCR assays [6,7], reverse-transcription PCR (RTPCR) [8] and real-time PCR (qPCR) using mainly the TaqMan® technology [9,10]. They are targeting genes such as iap, prfA and hlyA involved in L. monocytogenes pathogenicity [11] and are therefore specific for this species.

In the present study, culture method and real-time PCR molecular detection of L. monocytogenes were compared and evaluated for the best detection method.

MATERIALS AND METHODS

Clinical samples, culture and DNA extraction

Two hundred cervix swabs samples were collected from pregnant women in seventh and eighth month of pregnancy which referred to the Tehran Women Reference Hospital. The swabs transfer to Lister laboratory (Tehran, Iran) in 2ml transport medium. 1ml was used for DNA extraction directly according to standard procedures [12]. Remaining sample keep in refrigerator for cold enrichment for 4 weeks and then cultured on Listeria selective ager (HiMedia Company, India). A strain of L. monocytogenes (PTCC 1298) was purchased from the Persian Type Culture Collection (PTCC) and used as positive control of culture and assessing primer specificity in PCR.

Primer designing, in silico validating and conventional PCR

PCR primers were designed according to the L. monocytogenes specific *hly* gene encoding listeriolysin [13]. All the hly sequences were obtained from the GenBank database (http://www.ncbi.nlm.nih.gov) and were aligned using Clustal W [14]. A specific primer was designed based on consensus sequence with AlleleID software (Premier Biosoft, Palo Alto, USA) and assessed the specificity of primer sequences in silico via NCBI-BLAST (http://blast.ncbi.nlm.nih.gov).

The specificity of the primers was tested using a strain of L. monocytogenes (PTCC 1298) as positive controls. The PCR reaction mixture included 12.5 µl master mix of Taq DNA polymerase with $MgCl_2$ (Ampliqon, Denmark), 1 µl each of the forward and reverse primers (stock concentration, 10 pmol/ µl), 10 ng of template DNA, and sterile distilled water to a 20 µl total final volume. The PCR thermal profile was as follows: initial denaturation at 94°C for 4 min, 35 cycles consisting of denaturation at 94°C for 1 min, primer annealing at 63°C for 1 min, extension at 72°C for 30 s and a final elongation step at 72°C for 5 min. Thermocycling was performed on a Eppendorf Mastercycler® gradient personal thermal cycler (Germany). The sequences of specific primer were 5′-CGCAACAAACTGAAGCAAAGG-3′ (forward) and 5′-TTGGCGGCACATTTGTCAC-3′ (reverse) which resulted 210bp amplicon.

Real-time PCR assay

Real-time PCR was optimized by hot-start condition using AccuPower® *2X* GreenStar Master mix solution (Bioneer, South Korea). Reactions were carried out in 0.2 ml Opaque White 8-strip, Low Profile PCR Tube ((Bioneer, South Korea) using Exicycler™ 96 Quantitative Real-Time PCR System (Bioneer, South Korea). Each reaction contained various volumes of template DNA (according to their concentration), 25 µl of GreenStar Master mix solution, 1 µl (10 pmol) of each primer and sterile water to bring the total reaction volume to 50 µl. Thermal-cycling parameters were as follows: 94 °C for 5 min, followed by 40 cycles of 94 °C for 30 s, 63 °C for 30 s, and 72 °C for 45 s. Fluorescent signal was collected at the extension step. At first, real time PCR-amplifications were performed with a range of concentrations of the L. monocytogenes genomic DNA. The specificity of real time PCR was evaluated using the melting temperature (Tm) calculated from the melting curve of the PCR product, which was obtained after completion of the PCR cycles. The Tm peak of the PCR product was calculated based on the initial fluorescence curve (F/T) by plotting the negative derivative of the fluorescent signal over temperature versus temperature (-dF/dT versus T).

RESULTS AND DISCUSSION

Optimization and specificity of real time PCR

PCR primers were designed based on L. monocytogenes specific hly sequences obtained from the GenBank database. In initial experiments to evaluate primer specificity using conventional PCR [12], the amplicon with the expected size (210 bp) were amplified for tested L. monocytogenes strain (Fig.1)

For real time PCR assay, the melting temperatures of the primers (Tm = 63) was lower than the annealing and extension temperatures of the standard shuttle cycle. The results of real time PCR showed positive signals (CT values) for L. monocytogenes. The average melting temperature of the amplified products was 83.2 ± 0.3 _C (Fig. 2). Fluorescence signals for the negative control strains were significantly lower than the threshold level. Tree genomic DNA concentration was tried for assessing of sensitivity of real time PCR. The concentration of DNA was 1ng, 100 pg and 10 pg. As showed in figure 3, the CT values for every DNA concentrate were 18, 22 and 30 respectively.

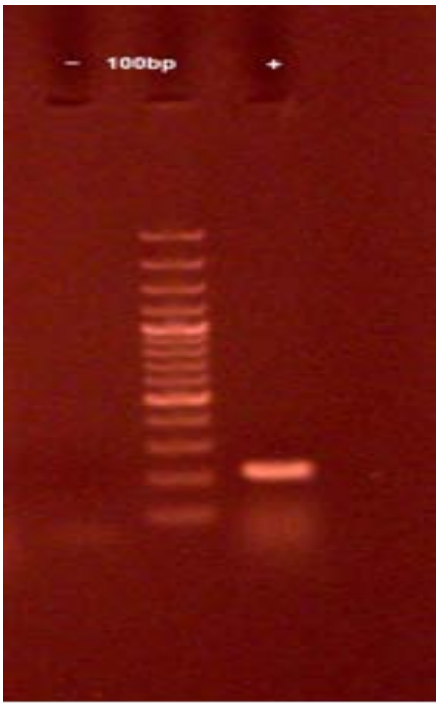

Fig.1: The result of conventional PCR was showed a specific band (210 bp) in compare of 100 bp ladder and negative control

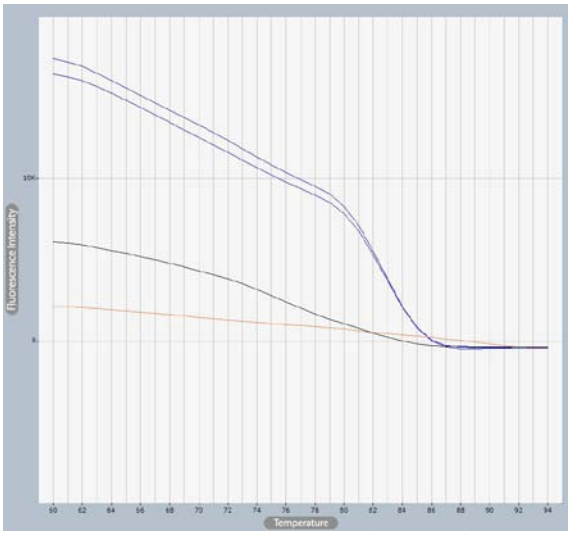

Fig. 2: The average melting temperature of the amplified products was 82.2 ± 0.3

The result of culture and real time PCR methods were showed of 200 samples, 28 (14%) and 41(20.5%) were positive, respectively.

The major advantage that molecular techniques offer over conventional methods is that they are based on differences within the genome and do not rely on the expression of certain antigenic factors or enzymes to facilitate identification. They are extremely accurate, reliable and some can be performed in the same time frame as immunoassay methods. There is a wide range of molecular methods available for the identification and characterization of *Listeria*, like conventional PCR methods.

PCR-based methods have been suggested as viable alternatives to laborious culture-based methods for the detection of L. monocytogenes, but require time-consuming

post-PCR analyses of PCR products, typically by gel electrophoresis. Therefore, a real time PCR method that eliminates post-PCR analysis was introduced [15-19]. However, the real time PCR method still requires the application of pre-PCR enrichment culture (24–48 h) [20] to remove PCR inhibitors from the clinical samples. In this study, we developed a simple, rapid procedure to direct real time PCR. All of 13 false negative via culture, were positive in PCR technique. So, molecular method was more sensitive and recommended for applying. Moreover, the results showed high prevalence of carrier women in this time. Therefore, public screening was recommended for pregnant women.

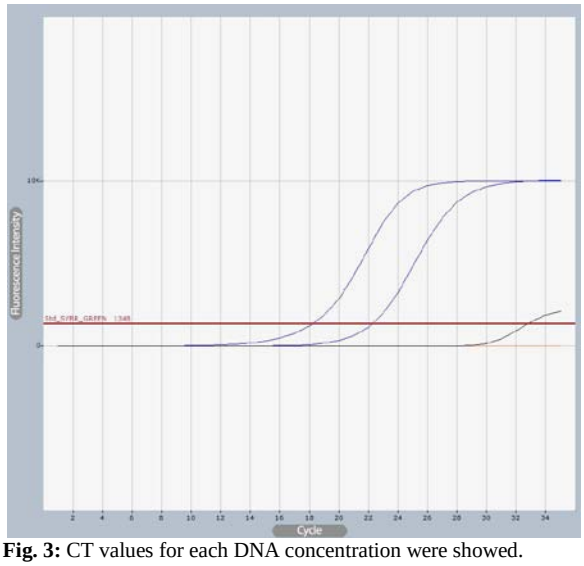

Fig. 3: CT values for each DNA concentration were showed.

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