

## Development of a Multiplex PCR Assay for Detection of *Listeria monocytogenes*, *Shigella* and *Escherichia coli* O157:H7 in Raw Milk

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To establish a multiplex PCR method and to apply multiplex PCR for simultaneous detection of *L. monocytogenes*, *S. flexneri* and *E. coli* O157 in milk. According to DNA sequences of the *hly* gene of *L. monocytogenes*, *ipaH* of *Shigella* and the *HlyA* gene of *E. coli* O157:H7, three pairs of specific primers were screened and designed to compose optimized system and conditions of multiplex PCR. The three expected sizes were 360bp for *L. monocytogenes*, 512bp for *Shigella* and 240bp for *E. coli* O157:H7. The detection sensitivity of multiplex PCR were above  $10^2$  cfu/ mL in raw milk. The multiplex PCR took 6-8 hours to detect a sample. The results of the experiments demonstrated that the multiplex PCR assay was rapid, simple, sensitive and specific, which established important foundation for simultaneous detection for these three bacteria in raw milk.

**Key words:** Multiplex PCR, *L. Monocytogenes*, *S.flexneri*, *E. coli* O157:H7.

In recent years, *L. monocytogenes* has been widely considered as an important zoonotic pathogen, which spread mainly through milk and its products. Although *listeriosis* is less common than other foodborne diseases, it remains a public health concern due to its high mortality rate (28%)<sup>1</sup>. *Shigella spp* are known as the infectious diarrhea pathogens, which is a kind of highly contagious and serious harm intestinal pathogen. About 160 million people were infected every year and about 1million 100 thousand were dead from this disease, mostly children under five years old who drunk the milk and its products<sup>2</sup>. *E. coli* O157:H7, has a characteristic rapid growth rate even under severe conditions that can easily compromise food safety

and contribute to health hazard<sup>3</sup>. So it is very important to detect these three pathogens by advanced detection technology.

The traditional colony culture and biochemical methods are effective and reliable but can take hours or days and are accompanied with laborious steps. Such drawbacks can be reduced if genetic and immunological approaches are employed because these methods are sensitive and reproducible compared to the conventional methods<sup>4</sup>. However, these streamlined alternative methods still require multiple reaction steps, long reaction time, well-equipped facilities, and well-trained person as well as a pre-enrichment step resulting in a 20- to 24-h turnaround time. Moreover, these traditional methods have some degree of false negative rate and false positive rate<sup>5</sup>. So these methods are difficult to fit for detecting these three kinds of bacteria in milk. The multiplex PCR technique which has the advantages of high specificity, high sensitivity, simple operation and low cost can easily solve these

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problems. The PCR technique has been used to detect *L. monocytogenes*, *S. flexneri* and *E. coli* O157:H7 in raw meat and raw milk, respectively<sup>6,7</sup>. However, to our best knowledge, it has not been seen the simultaneous detection of these three kinds of pathogenic bacteria in raw milk was by multiplex PCR.

In this study, to simplify the operational process and to raise the sensitivity and specificity, the multiplex PCR methods were developed to detect the three pathogenic bacteria: *Listeria monocytogenes*, *S. flexneri* and *E. coli* O157:H7 in raw milk.

## MATERIALS AND METHODS

### Strains

The bacterial strains used in this study for developing PCR method were *Listeria monocytogenes* ATCC13932, *Shigella flexneri* CMCC51572 which both were obtained from Centers for Medical Culture Collection (CMCC, Beijing, China) and *E. coli* O157:H7 CICC21530 which was purchased from China Center of Industrial Culture Collection (CICC, Beijing, China).

### Agent

Taq DNA polymerase (5 U/μL); 10 × PCR Buffer (Mg<sup>2+</sup> free); MgCl<sub>2</sub> (25 mmol/L); dNTP Mixture (2.5 mmol/L); 100 bp DNA ladder Marker; GOLDVIEW<sup>TM</sup> colorant agent; agar bacterial genomic DNA extraction test kit (all of these agents were bought from Sangon, China).

### The strains cultivation and genomic DNA extraction

These three strains of bacteria were inoculated in the TSB-YE medium at 200 r/min shaking table for 12h and they made the concentration of the bacteria reached to 10<sup>8</sup> cfu/mL. 1ml of each bacterium liquid was taken into the 1.5ml centrifuge tube and centrifuged for 5 min. The bacterium genomic DNA was extracted by bacterial genomic DNA extraction test kit and was as the multiple PCR positive templates.

### Primers designed

Based on the DNA sequences of the *hly* gene of *L. monocytogenes*, *ipaH* of *Shigella* and *hlyA* gene of *E. coli* O157:H7, three pairs of specific primers were designed by Primer 5.0. (Table 1)

### Establishment of the multiplex PCR system

The extracted genomic DNA from each strain was as the template and each pair of primers were used to amplify the gene respectively by routine PCR method. The multiplex PCR was carried out after finding the same condition and uniformed the conditions at the step of the routine PCR method. The smaller affecting factors were determined and the larger affecting factors were readjusted by many times of experiments. Each primer was added the same quality at the early multiplex PCR<sup>8</sup>.

### PCR products analysis

PCR products were analyzed by electrophoresis with 1.5% (wt/vol) agarose gel containing 1.0 of GOLDVIEW<sup>TM</sup> per mL. These were visualized with gel imaging system and photographed. DNA molecular size standards (100 bp ladder, Gibco/BRL) were included in each agarose gel electrophoresis.

### Artificially contaminated raw milk and filtration pre-treatment

The same concentration of the three kinds of pathogenic bacteria were added into the 200ml of raw milk and made the final concentrations of each bacteria 10<sup>0</sup>, 10<sup>1</sup>, 10<sup>2</sup>, 10<sup>3</sup>, 10<sup>4</sup>, and 10<sup>5</sup> cfu/mL, respectively. And then the milk was centrifuged at 8000g for 10min and removed the upper body fat carefully. After that, in order to destroy the milk casein glue and to make the milk limpid, each sample was added the 30ml of 50% EDTA-2Na (volume ratio), 37°C water bath for 30 min<sup>9</sup>.

### The contaminated milk filtered and detected by multiplex PCR

The limpid raw milk was filtered through the Seitz filter and the filter membranes were cut into pieces and placed into a dry sterile flask. The 20ml physiological saline was added into the flask and then put them on the vortex alternator to vibration for 2 min. Then the eluant was centrifuged at 10000g for 5min and the supernatant was discarded. The cell precipitate was resuspended with the 1mL of sterile distilled water and then centrifuged at 10000g for 5 min, removing the supernatant and obtained thallus. The bacterium genomic DNA was extracted by bacterial genomic DNA extraction test kit and as the multiple PCR positive templates. The multiplex PCR was carried out and the PCR products were analyzed by gel imaging system.

## RESULTS

### Establishment of the multiplex PCR system

In amplification conditions, the annealing temperature had the greatest impact on the reaction. The multiplex PCR was carried out at different annealing temperatures with the same amount of each primer (the annealing temperature was set at 52°C-62°C). It was found that the best amplification temperature was at 58°C. At this annealing temperature, the concentration of the primer was optimized. When the equivalent concentrations of the three pairs of the primer were at 100, 150, 200, 250, 300 µmol/L, respectively, it was found that the best amplification was at 150 µmol/L. But the PCR production was not uniformed at this amplification conditions. According to the gel gray scale, increase of concentration of the primer which amplified bands was weak. Based on the best annealing temperature and the concentration of the primer,

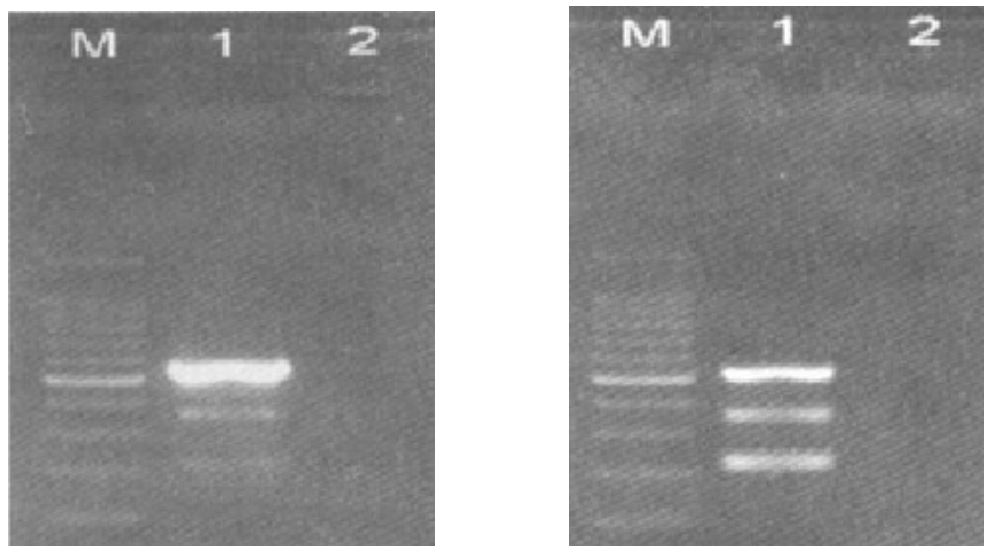
the amount of enzyme was optimized. When the amount of enzyme was at 10, 7.5, 5, 2.5, 1.25U, it was found that the best amount of enzyme was at 5U. The results of the gel electrophoresis of the objective DNA by the multiplex PCR were showed in Fig.1.

In this study, the multiplex PCR system and the conditions were as followed:

- (1) The optimization of the multiplex PCR system: 10×PCR buffer: 5 µL, dNTPs: 600 µmol/L, the concentration of the primer: 250 µmol/L for *L. monocytogenes*, 125 µmol/L for *Shigella* and 250 µmol/L for *E. coli O157*, Taq enzyme: 5U, DNA template: 4 µL, reaction system: 50 µL.
- (2) The amplification reaction conditions: Thirty-five temperature cycles consisted of 5 min at 94°C, followed by 45 s at 94°C, 45 s at 58°C and 60 s at 72°C, and repeated 35 cycles. The final cycle was followed by incubation of the reaction mixture for 5 min at 72°C.

**Table 1.** PCR primers for the target gene

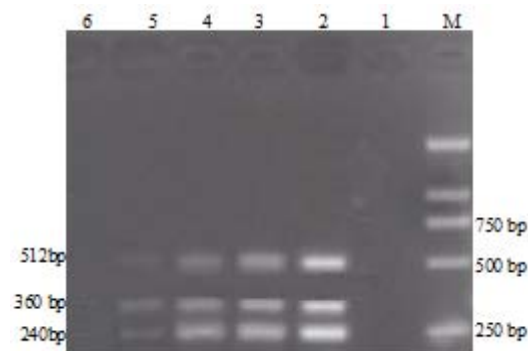
The bacteria	Primers	Sequence of the primers ( 5' to 3')	Size (bp)
<i>L.monocytogenes</i>	hly	F: TCTGTCTCAGGTGATGTAGA R: TGC GTTACCTTCAGGATCAT	360
<i>S.flexneri</i>	ipaH	P: AATGGCTGGAAAACTCAGTGC R:CTTATACTTCTGCTCTTCTGCC	512
<i>E. coli O157</i>	Hly A	P: AACAGGAGTTGTCAGTGGGGAAGC R:CCACGTGATTGACGTATAGTT	240



**Fig. 1.** The results of the multiplex PCR before and after optimization M DL 1500 Marker, 1: the amplified fragment by using three pairs of optimized primers for these three strains; 2: Negative control

### The detection sensitivity of the three bacteria in raw milk

Adding same number of the three bacteria in the raw milk at different concentrations. The pollution gradients were  $10^5$ ,  $10^4$ ,  $10^3$ ,  $10^2$ , and  $10^1$  cfu/mL. After the pre-treatment, Seitz filter filtration, the genomic DNA extraction, the results of the gel electrophoresis of the objective DNA by the multiplex PCR was showed in Fig.2. And the detection sensitivity of the multiplex PCR can reach above  $10^2$  cfu/mL.



**Fig. 2.** The sensitivity of multiplex PCR for *L. monocytogenes*, *S. flexneri* and *E. coli* O157:H7 M DL 2000Marker, 1: Negative control, From 2 to 6:  $10^5$ ,  $10^4$ ,  $10^3$ ,  $10^2$  and  $10^1$  cfu/mL

### DISCUSSION

The primer design was critical in multiplex, which determined the success of the PCR reaction. Primer design must be specific. The pathogenicity of *L. monocytogenes* is connected with a number of virulence factors, such as hemolysin, internalin, P60 protein, and so on. The P60 protein is encoded by *iap* gene, but it is owned by all the species of *Listeria*. The *hly* gene which encoded the hemolysin is specific for *L. monocytogenes*<sup>10, 11</sup> and was selected for specific PCR amplification in this study.

The *ipaH* gene was selected for designing the primer because it is specific, it is present in all species of *Shigella* and resides in the chromosome and invasive plasmid by multiple copies and did not lose with the passage<sup>9, 12</sup>.

However, there was not a gene in *E. coli* O157: H7, which not only was responsible for its toxicity but also could be distinguished from other species<sup>13</sup>. The *hlyA* gene, which was in all of the

pathogenic O157: H7 strains, was selected for the purpose of detecting *E. coli* O157: H7. But the *hlyA* gene was also contained in several other serotypes of *E. coli*. When it was positive in the samples, it needed to be further corroborated by some other means.

Many factors could affect multiplex PCR, such as different primer, template, primer concentration, Tag enzyme concentration,  $Mg^{2+}$  concentration and cycling parameters<sup>14</sup>. Because the primer design and system ratio was reasonable, the factors that significantly affected the multiplex PCR were the suitable concentration of the primers, Tag enzyme concentration and annealing temperature when optimized the multiplex PCR system. When the same concentration of the primers was added, the amplification production was not equal. It would make the amplification production more equal by increasing the primer concentration which production was less. The other effect on the amplification production was the Tag enzyme concentration. Because the Tag enzyme contained a high concentration of glycerin, it resulted in the imbalance of the amplification production. The most efficient concentration of enzyme was about 2U/25  $\mu$ L reaction volume, so after reducing the amount of enzyme used, the amplification production by multiplex PCR reached a more homogeneous state.

In order to optimize the annealing temperature, different annealing temperatures were tested. When the temperature was at 59 °C, each primer could amplify the objective fragment respectively and reached to the highest efficiency. In this test, the single annealing temperature was used. But in some cases, even in the same condition, the objective fragment could be amplified by conventional PCR, but not every objective fragment could be amplified. When this condition occurred, the gradient PCR could be used to optimize the annealing temperature<sup>15, 16</sup>.

In our study, the 0.22  $\mu$ m of microporous membrane was as the bacterial filter used for the volume of the bacteria cell about  $(0.4-2) \times (1-3)$  and the temperature was set at about 37°C for filtration. If the temperature was too high, the viscosity of the solution would be decreased and the diffusion would be increased

The whole detection could be finished in 6-8 h and the detection sensitivity of the three

pathogenic bacteria in raw milk could reach above  $10^2$  cfu/ mL. But for the bacteria which were too few in the testing sample, this method would give false negative results.

## CONCLUSION

To sum up, a systematic and rapid method for detection and identification of *L.monocytogenes*, *S.flexneri* and *E. coli O157:H7* in raw milk at the same time was developed in this study. The greatest advantage of the multiplex PCR detection method for these three pathogens was its speed of detection. This method was simple, fast, specific and sensitive for detecting these three pathogens in raw milk.

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