Application of Agilent 2100 Bioanalyzer in Multiplex PCR for Detection of Foodborne Pathogens*

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Abstract—To explore the feasibility of using Agilent 2100 Bioanalyzer in the detection of the three kinds of food-borne pathogens, three pairs of primers were designed according to invA gene of Salmonella, ipah gene of Shigella and inv gene of Yersinia pseudotuberculosis, and then the single PCR was performed to detect specificity. Those genes were amplified by Multiplex PCR technique using specific primers, and the optimum of multiplex PCR reaction conditions. The multiplex PCR detection method which provided a basis for further research on developing the food-borne pathogens detection kit was very practical. Agilent 2100 Bioanalyzer combined with Multiplex PCR was specific, accurate and sensitive for foodborne pathogens Salmonella, Shigella and Yersinia pseudotuberculosis detection.

Keywords—Food-borne Pathogens; Multiplex PCR; Agilent 2100 Bioanalyzer; Testing Method

I. INTRODUCTION

Salmonella, Shigella and Yersinia pseudotuberculosis contamination is one of the most commonly reported causes of food-borne disease in the world[1]. Detection of pathogenic bacteria present mainly relies on conventional microbiological methods, which normally takes 4 ~ 7 d time. Another applications, such as immunoenzyme test, immunofluorescence assay, gene probe are relatively low sensitivity, poor specificity, and they are difficult to promote[2]. Agilent 2100 Bioanalyzer is an analytical system of nucleic acid for laboratory, which is industrial and with the biological chip as the base, and which overcomes the traditional limitations of gel electrophoresis[3-5]. Multi-PCR in the same PCR reaction system by adding more pairs of primers can amplify a number of target gene, and which overcomes the traditional limitations of gel electrophoresis[6-7]. In pathogen detection, the multiple PCR can not only enhance the detection efficiency, but also save the cost of the detection. In this paper, as for the common food-borne pathogens, such as Salmonella, Shigella and Yersinia Pseudotuberculosis were detected by the multiple PCR and Agilent 2100 Bioanalyzer at the same time. The aim of the study was to find and verify a new pathogens testing method.

II. MATERIALS AND METHODS

A. Materials and Reagents

Pathogens: typhimurium(GIM1.237), Salmonella paratyphi-A(GIM1.235), Salmonella paratyphi-B(GIM1.224), Salmonella spp.(CGMCC1.1552), Shigella spp.-A(GIM1.238), Shigella spp.-B(GIM1.239), Shigella spp. - C(CGMC51252), Yersinia Pseudotuberculosis(GIM1.267), bought from Guangzhou province microorganism fungiform conserve center and Chinese microorganism fungiform conserve center. Primers of PCR were showed as table 1.

<table>
<thead>
<tr>
<th>Pathogens</th>
<th>Target gene</th>
<th>PCR Primer sequences (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salmonella spp.</td>
<td>invA gene</td>
<td>Sal-3a, TATGCGCCACGTCCGCGAA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sal-4a, TCGCAGCGTCAAGGAAACC</td>
</tr>
<tr>
<td>Shigella spp.</td>
<td>ipah gene</td>
<td>Shi-1a, CTGGACGCGTCTTGCAGTA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Shi-2a, CGACCCACCTCTGAGATGA</td>
</tr>
<tr>
<td>Yersinia</td>
<td>inv gene</td>
<td>YP-3a, CGTGCTGATGCACCAGAT</td>
</tr>
<tr>
<td>Pseudotuberculosis</td>
<td></td>
<td>YP-4a, TGGTCACCTGGACCTGTG</td>
</tr>
</tbody>
</table>

B. Experimental procedures

1) Template Preparation

Bacterial DNA was extracted by Chelex-100 method. Chelex-100 was used as an ion-chelating agent, composed of styrene and Diethylene benzene. Its suspension in the alkaline environment (pH10 ~ 11) and under the conditions of 100 ℃ could lead to the breakdown of cell membranes and the viability and release of DNA[8,9].

(1) The sample was added to 1.5 mL centrifuge tube with 500 μL water, then was vibrated Violently, placed at room temperature for 15 min.

(2) Centrifuged for 3min at 13,000 rpm, the supernatant was removed, the pellets were collected. (Distilled water could be used repeatedly, if necessary, to wash sediment until colorless or very little pigment).
(3) The pellet was suspended in 200 μL 5% Chelex-100 solution (5% Chelex-100 which was the suspension, must be shook before used, so that particles could be suspended), shook repeatedly, then stored for 30 min at 56 °C.

(4) Shook and kept for 8 min at 100 °C, then shook and centrifuged for 3 min at 13,000 rpm, the supernatant was used for PCR amplification, or preserved at 4 °C.

2) Optimum of triple-PCR reaction conditions

Salmonella, Shigella and Yersinia Pseudotuberculosis were used as templates, to optimize the reaction conditions of the multiple PCR. The initial reaction conditions (25 μL) included 10 × PCR buffer 2.5 μL, 25 mmol / L Mg2+ 1.6 μL, 2.5 mmol / L dNTP 2.0 μL, 2.0 μmol / L three pairs of primer 1.0 μL separately, 3 U / μL Taq DNA polymerase 0.5 μL. And then had optimization studies on the PCR annealing temperature, Mg2+ concentration, primer concentration.

3) Specific primers

Standard strains from Salmonella, Shigella and Yersinia Pseudotuberculosis were used as templates for DNA-specific PCR. The optimized results of the above PCR considered as reaction conditions.

4) Triple PCR sensitivity

Three types of standard strains (Salmonella, Shigella and Yersinia pseudotuberculosis) were cultured to logarithmic phase. The LB agar plate was used to count the strains, 10^8 cfu, were diluted with ultra-pure water to 10^8 times. Centrifuged 7,000 rpm 20 min, Chelex-100 was used to extract DNA. PCR items: 94 °C, 5min; 94 °C, 30s; 63 °C, 50s; 72 °C, 30min; 30cycles; 72 °C, 5min.

5) Agarose gel electrophoresis analysis

6 μl PCR production above was used for 1.5% agarose gel electrophoresis, 60V, 1h, and then results were taken a picture and analyzed amplified production according to the length relevant production.

6) Chip Detection and Analysis

Everything was done according to DNA 7500 LabChip Kit introductions. Gel and dyestuff compound were prepared as: 400 μl gel ground substance mixed with 20 μl concentrated dyestuff was filtrated by centrifugal filtrated machine. Gel and dyestuff compound were pouring in the chip and 5 μl marker were added to each sample well. After PCR amplification 1 μl of each reaction was loaded into 12 wells chip (DNA 7500 LabChip Kit), then 1 μl ladder was added to the appointed ladder well. Finally during vortexing chips were mixed well and inserted in the Agilent 2100 Bioanalyzer within 5 minutes for detection and analysis automatically.

III. RESULTS AND ANALYSIS

A. Optimum of triple-PCR reaction conditions

Shigella spp. was used as template. PCR annealing temperature, Mg2+ concentration and primer concentration were optimized. As figures 1, 2 and 3 showed that the best annealing temperature for PCR reaction was 63 °C, the optimum Mg2+ concentration was 1.6 mmol / L, the best primer concentration of Salmonella, Shigella and Yersinia pseudotuberculosis was 120 nmol / L, 80 nmol / L and 80 nmol / L, respectively.

Fig. 1 Effect of anneal temperature for multiplex PCR

Fig. 2 Effect of Mg2+ concentration for multiplex PCR

Fig. 3 Effect of primer concentration for multiplex PCR

B. Primer Specificity

In the above-mentioned conditions optimization of PCR, extracted DNA 14 strains of bacteria was amplified with three pairs of designed primers, special character of primers was detected. The results showed that the length of four kinds of Salmonella spp. amplified fragment were 275 bp, the length of three kinds of Shigella spp. amplified fragment were 610 bp, the length of Yersinia Pseudotuberculosis amplified fragment were 440 bp. Primers had no cross-reaction.
C. The sensitivity of PCR triple

10^8 cfu of Salmonella, Shigella and Yersinia Pseudotuberculosis, was diluted by 10-fold dilution gradient with ultrapure water respectively. The bacterial content for the reaction were 10^6 cfu, 10^7 cfu, 10^8 cfu, 10^9 cfu, 10^10 cfu, 10^11 cfu, 10^12 cfu, 10^13 cfu, and 10^14 cfu respectively, with using Chelex-100 to extract DNA. Then the triple-PCR was used under the optimized conditions, the product of electrophoresis results were showed in figure 7, Figure 8 and Figure 9. In gel electrophoresis, after triple-PCR, the sensitivity of Salmonella, Shigella and Yersinia Pseudotuberculosis were 10^3 cfu, 10^3 cfu and 10^3 cfu ingel electrophoresis.

D. PCR product detection and analysis with Bioanalyzer

1) Comparison of Accuracy and Repeatability between Bioanalyzer and Agarose Gel Electrophoresis

As figure 4, figure 5, figure 6 figure 10 and figure 11 showed that the detection methods were able to detect the fragments. Otherwise, there was a little error between the size through testing and the actual size of the fragments. In figure 11, by comparing the length of the fragment between test and actual, the length of fragments detected by Bioanalyzer was 278 bp, 452 bp, 623 bp, respectively. The actual length was 275 bp, 440 bp and 610 bp, respectively. Error rate between them was 1.8%, 2.7% and 2.1%, which was within the report margin of error of less than 5%. The results Agarose gel electrophoresis could only be used as a visual estimate of the size of fragment, and the error rate was 15%. On the right of figure 11, there was a gel-like image corresponding to the peaks, which made it easier to be observed and analyzed.
In this study, by optimizing the PCR reaction conditions, the triple-PCR system about rapid detection of *Salmonella*, *Shigella* and *Pseudotuberculosis* had the merits of specificity, sensitivity, time saving and steps simplifying. The minimum detection limit could reach 1 cfu. The entire detection time was less than 20 h, with a strong application. After further standardize, test kits will be developed so as to lay the foundation for the popularization and application of food-borne pathogenic micro-organisms detection kit. Bioanalyzer combined with multi-chip PCR method had an important exercisable value in detecting and identification of food-borne pathogens and was worth popularizing.

IV. DISCUSSIONS

PCR detection for pathogens is rapid, sensitive and specific, respectively in the process of detection the environment of the sample is important for detection. Foods are complex substances. Varieties of factors which have an impact on food safety must be taken into account. In the actual food testing, there is a very small number of positive samples which can be used to confirm in the conventional methods. On the other hand, the pathogen content of the samples are generally small and a large number of PCR inhibitors exist in the samples, which are severely affected the PCR amplification results, false-negative results. Therefore, to find effective methods of sample pre-treatment, the bacteria-rich samples, the existence of the samples to remove PCR inhibitors, can greatly increase the food-borne pathogens in the practicality of PCR detection methods[10]. Therefore recommended that in practice, firstly used multiple PCR method for screening samples, and then combined with the national standard method to verify, thereby reduced the workload and improved efficiency.

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