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Optimization of Multiplex PCR for Multiple Locus Variable Number Tandem Repeat Analysis for *Salmonella typhimurium*

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Abstract

Salmonella typhimurium infects an estimated 1.4 million in the United States and kills over five hundred annually. The current way of categorization, Pulse Field Gel Electrophoresis (PFGE) and antibiotic sensitivity testing, lack the ability to distinguish between some closely related strains. We are attempting to optimize a new technique of categorization, Multiple Locus Variable number tandem repeat Analysis (MLVA). We made changes to temperature and concentration variables within the multiplex PCR procedure to streamline the identification process. By optimizing the multiplex PCR procedure we intend to create more consistent amplification of the loci used in MLVA analysis, which will improve efficiency in differentiating *S. typhimurium* isolates.

Disciplines

Chemistry

Optimization of Multiplex PCR for Multiple Locus Variable Number Tandem Repeat Analysis for *Salmonella typhimurium*

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Abstract

Salmonella typhimurium infects an estimated 1.4 million in the United States and kills over five hundred annually. The current way of categorization, Pulse Field Gel Electrophoresis (PFGE) and antibiotic sensitivity testing, lack the ability to distinguish between some closely related strains. We are attempting to optimize a new technique of categorization, Multiple Locus Variable number tandem repeat Analysis (MLVA). We made changes to temperature and concentration variables within the multiplex PCR procedure to streamline the identification process. By optimizing the multiplex PCR procedure we intend to create more consistent amplification of the loci used in MLVA analysis, which will improve efficiency in differentiating *S. typhimurium* isolates.

Introduction

Salmonella enterica serotype typhimurium is a pathogenic Gram-negative bacteria that is predominately found in the intestinal lumen of animals. This bacteria is toxic to humans largely because of the outer membrane being coated by lipopolysaccharides or LPS which protect the bacteria from the environment. The LPS contains an O-antigen, a polysaccharide core, and lipid A, which is the connects it to the outer membrane. Lipid A is made up of two phosphorylated glucosamines which are attached to fatty acids. These phosphate groups determine bacterial toxicity. The O-antigen, being on the exterior of the LPS complex is responsible for the host immune response. *S. typhimurium* has the ability to go through acetylation of this O-antigen, which modifies its conformation and allows for evasion of the host immunes response (1).

Figure 1: Salmonella typhimurium (shown in red) invading surrounding cells (2).

The polymerase chain reaction (PCR) is a technique used in molecular biology to amplify small amounts of DNA to high enough levels to perform scientific research, generating thousands to millions of copies of a particular DNA sequence. This method depends on a process called thermal cycling, a procedure consisting of cycles of repeated heating and cooling of the reaction to cause DNA melting and replication. Primers, which are short DNA fragments, that contain sequences complementary to the target region along with a DNA polymerase (which the method is named after) are key components to enable selective and repeated amplification. As PCR progresses, the DNA generated is itself used as a template for replication, setting in motion a chain reaction in which the DNA template is exponentially amplified. PCR can be extensively modified to perform a wide array of genetic manipulations. This experiment utilizes a specific type of PCR called Multiplex PCR. Multiplex PCR is a variant of PCR which allows for simultaneous amplification of several targets of interest in a single reaction by using multiple primers (3).

Multiple Loci Variable Number Tandem Repeat Analysis (MLVA) is a method used to analyze DNA, commonly used for analysis of pathogenic bacteria. The method allows for identification of repeating units at each loci present and determines their concentration within the loci (4). For this experiment, this method is used to determine what concentrations are effective to use for multiplex PCR to amplify each of four loci of Salmonella *typhimurium* (ST3, ST5, ST7, and STTR10) to a similar level by varying annealing temperature and concentration of the loci prior to PCR.

Materials and Methods

Materials

PCR primers and S. *Typhimurium* samples were provided by the Center for Disease Control. Illustra puReTaq ready-To-Go PCR beads and agarose gel components were provided by the Boise State Chemistry Department (5).

Methods

DNA preparations
An S. *Typhimurium* DNA template was extracted from samples for amplification of select loci by PCR. Three 100µL aliquots of water were inoculated with one colony from three strains of S. *Typhimurium.* The samples were placed in a 100ºC water bath for 10 minutes and then centrifuged at 10,000 RPM for 10 minutes. The supernatant, containing the DNA, was collected and stored at -20ºC (5).

Individual Loci PCR
Individual loci PCR amplification with varying annealing temperatures were tested to obtain a temperature appropriate for all loci being amplified. This amplification was done in accordance with the CDC proposed primer and template concentrations shown in Table 1. The PCR cycle consisted of a 5 minute 95ºC hot start then 35 cycles of the following.

- 94 ºC-20 seconds
- Annealing temperatures were varied, 59.1ºC, 60.8ºC, 64.4ºC, and 68.1ºC – 20 seconds
- 72ºC-20 seconds

Proceeding that a 5 minute 72ºC annealing stage and then a 4ºC hold were implemented.
Primer concentrations were optimized in a second PCR reaction. This PCR was run with the same cycle but with constant annealing temperatures, 60ºC. The primer concentrations were varied for loci ST3 and ST5 to obtain consistent banding. The concentrations ran for ST3 were .07µM, .14µM and .21µM, and for ST5 1.0µM and 1.5µM (5).

Multiplex PCR
Multiplex PCR was run with only three out of the four loci primers present. The ST5, ST7 and STTR10 loci were together at the suggested concentrations of the CDC (Table 1). The PCR cycle was the same as the single loci amplification with the exception of a constant annealing temperature of 60ºC (5).

Gel analysis
For the analysis of the PCR products agarose gels were ran. For the analysis of the single loci PCR amplifications a 1% gel was used, for the multiplex PCR reaction a 2% gel was used in conjugate with a fisher 1KB pre-stained ladder (Figure 4). 10µL of PCR product were loaded for each well, the gels and lain assignments can be seen in Figure 3 (5).

Table 1: Amplicon size obtained from multiplex PCR of loci and suggested primer concentrations from the CDC

Locus	CDC suggested primer concentration	Amplicon size
ST3	0.07	178 - 190
ST5	1.5	184 - 231
ST7	0.4	138 - 150
STTR10	0.03	382 - 413

Results

Individual Loci PCR

Individual amplifications of the ST3, ST5, ST7 and STTR10 loci were run to determine the proper annealing temperature and primer concentrations for multiplex PCR. The PCR products were analyzed via an agarose gel (Figure 2). The bands of the amplicons are in alignment with the expected size given by the CDC which can be found in Table 1. From the first PCR with varying annealing temperature, 60ºC was chosen to study the proper concentration of primers. Varying concentrations of primers were used for ST3 and ST5 loci, the PCR products were run on a 1% agarose gel and can be found in Figure 3.

Multiplex PCR

Multiplex PCR containing primers for ST5, ST7, and STTR 10 was conducted and the products were run on a 2% agarose gel. The band corresponding to the STTR10 locus had less than have the intensity of the bands corresponding to the ST5 and ST7 loci demonstrating a need for an increase in primer concentration. The ST3 locus was left out of the multiplex reaction due to an unknown concentration requirement.

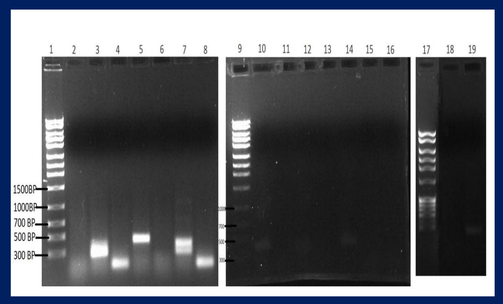

Figure 2: 1% agarose gel run with products from single loci PCR products demonstrating different annealing temperatures for the ST3, ST5, ST7 and STTR10 loci. Lanes 1,9 and 17 are Fisher 1KB ladders. Lanes 2,3,4,5 were run with an annealing temperature of 59.1ºC, lanes 6,7,8 and 10 with an annealing temperature of 60.8ºC, lanes 11,12,13 and 14 with an annealing temperature of 64.4ºC, lanes 15,16,18 and 19 with an annealing temperature of 68.1. All groups of four contain loci ST3,ST5,ST7 and STTR10 respectively.

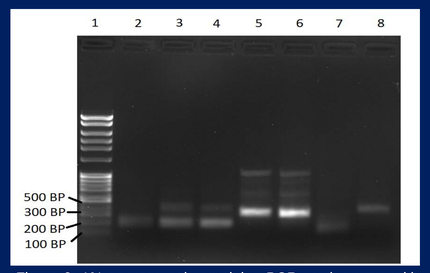

Figure 3: 1% agarose gel containing PCR products run with a constant annealing temperature of 60ºC and varying concentrations of primers for ST3 and ST5. Lane 1 contains a Fisher 1Kb ladder, Lane 2- ST3 primers at .07µM, Lane 3-ST3 primers at .14µM, Lane 4-ST3 primers at .21µM, Lane 5-ST5 primers at 1.0µM, Lane 6-ST5 primers at 1.5µM, Lane 7-ST7 primers at .4µM, Lane 8-STTR10 primers at .03 µM.

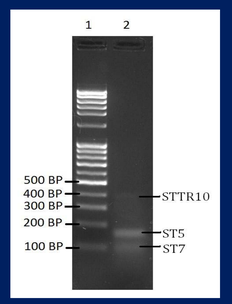

Figure 4: Multiplex PCR products with primers ST5, ST7 and STTR10 run on a 2% agarose gel. Lane 1-Fisher 1KB ladder, Lane 2-Multiplex PCR products.

Discussion

The optimization of a multiplex PCR procedure requires three different experiments, first the optimization of annealing temperatures for the multiple loci, second, the optimization of primer concentrations at the desired annealing temperature and third, the optimization of a multiplex PCR reaction with all reagents in the same solution.

With the first experiments of varying annealing temperatures during the PCR reaction cycle, it was found that the temperatures from 59.1-60.8ºC were appropriate for all four loci being amplified (Figure 2). Due to this data, an annealing temperature of 60ºC was chosen for all of the following PCR reactions conducted. The choice of a low annealing temperature could cause nonspecific binding of primers and give undesired products, It may be necessary to raise the annealing temperature to prevent this in the multiplex reaction.

The second optimization was done by changing primer concentrations in accordance with data collected at different annealing temperatures (Figure 2). Lane 2 had a weak band indicating a lack of amplification of locus ST3. Differing concentrations of ST3 primers were used in PCR and the strength of the bands can be observed in lanes 2-4 of figure 2. The concentration of .21µM gave a band strength that was congruent with the bands of the other loci. The ST5 amplicon in figure 1 lane 3 was too strong; the concentration was reduced and ran at 1.0 µM and 1.5µM in lanes 5 and 6 of figure 2. For future multiplex PCR ST3 primers will be prepared at .21µM and ST5 at 1.0µM to produce approximately equal concentrations of product.

Future Works

The future multiplex PCR reaction should work well with the concentrations and annealing temperatures outlined by the previous two experiments. There are possibilities of differing primer interactions, this could explain the extremely weak band produced by STTR10 in the multiplex reaction (Figure 3). More multiplex reactions will be required to perform proper optimization of this process.

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