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PCR Optimization of BOX-A1R PCR for Microbial Source Tracking of *Escherichia coli* in Waterways

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Different PCR parameters and conditions were investigated to identify the optimal conditions for improved reproducibility and intensity of BOX A1R PCR products. Four isolates of *Escherichia coli* of either gull or duck origins were cultured, washed, and used as whole cell PCR templates. BOX A1R PCR was performed using template from different growth phases, and by varying PCR annealing temperatures, and the presence/absence of bovine serum albumin (BSA). The results showed that the growth phases of the culture template did not impact either reproducibility or intensity of the PCR bands. Different PCR annealing temperature did affect the intensity of different molecular weight bands and the presence of different bands. Generally, high PCR annealing temperature produced more visible low molecular weight bands and low PCR annealing temperature produced high molecular weight bands. The addition of BSA appeared to have a negative effect on the intensity of the bands. Even though from the results optimal PCR conditions could be approximated from the results, there are other parameters and factors in the modified PCR protocol that should be investigated to further improve the PCR results for accurate microbial source tracking analysis.

Fecal contamination is linked to major health and hygiene concerns across the globe. Majority of the contamination in fresh-water sources is contributed by agricultural uses of manure, ineffective waste water treatment, and inappropriate residential and industrial developments (3, 5). For example, in Minnesota, 47% of the river area investigated for fecal coliform bacteria count exceeded the threshold value for safe swimming (2). In addition, more than 90% of the Minnesota River contained high level of fecal coliform counts, and these values were consistently increasing at alarming rates (2). In order to develop efficient control strategies against water-borne fecal coliform, identification of the source of contamination is crucial. A promising approach to identify bacterial contamination sources is the Microbial Source Tracking (MST), which uses patterns of molecular identifiers of *E. coli* strains to identify the source of microbial contamination. One of the current methods employs the BOX A1R primer for repetitive element sequence-based PCR (rep-PCR) to amplify the repetitive regions of bacterial genome. This primer was a special interest in many previous studies because it is found in many microbial genomes (6). The band profiles of the amplified repetitive regions are unique among species, or even between species, thus different species can be identified through their band patterns. However, BOX A1R PCR does not consistently yield robust high-resolution bands necessary for MST analysis (2, 6, 12).

In 2009, Lee and Wong (6) attempted to optimize the BOX A1R process by altering several different PCR parameters, such as using different template dilutions, restriction digestions, and addition of different bovine serum albumin (BSA) concentrations. Their results demonstrated that while the majority of alterations performed in the PCR parameters did not yield notable changes to the PCR results, the addition of BSA significantly improved the outcomes by producing more reproducible and intensified bands (6). Following, Zhu did a comparison of four template preparation methods for BOX A1R PCR to optimize the resulting bands' resolution and robustness: cell washing, alkaline lysis, heat lysis, and DNA isolation (12). That study found that three of the four methods, cell washing, heat lysis, and DNA isolation, had a notable impact on the reproducibility and resolution of the bands, while the alkaline lysis did not (12). That study suggested while most methods work, the preparation by washing methods would be most useful because it was an inexpensive and rapid way of preparing high-purity samples for BOX A1R PCR.

Although the preceding experiments improved the BOX A1R PCR results, the results were still unsatisfactory in terms of reproducibility and resolution for MST analysis. In the study of Lee and Wong's, the resulting band patterns were not consistently reproducible and were often too faint for MST analysis (3, 6). While the study by Zhu was able to improve the

robustness and reproducibility of the BOX A1R PCR, the intensity and resolution of the band patterns were still inadequate (12). Both studies suggested several parameters of the PCR procedures could be further tested for optimization, including changing the primer concentration to avoid formation of primer dimers, changing the annealing temperature and incorporating of BSA into the PCR protocol. Another study had also investigated in the influence the growth phase of the template cell culture on the intensity of the PCR bands (1). One of the major physiological differences in exponential growth and stationary phases is the thickness of the peptidoglycan layer, which may range from 1.5 nm at growth phase to 15 nm at stationary phase (11). Although in the study by Abolmaaty *et al.* (1) the two different growth phases did not demonstrate any difference in the amount of DNA extracted via enzymatic lysis, this could be a factor in whole-cell PCR procedure, in which extraction is based on treatment with high salt concentration. The experiment investigated the efficacy of improving band intensity and reproducibility by adjusting the rep-PCR parameters to make the PCR results useful for detailed analysis.

MATERIALS AND METHODS

Media preparation. MacConkey agar was prepared from premixed LB MacConkey Agar powder (BBL, Lot# 8113870, 0194). Lysogeny broth was prepared by dissolving 2.5 g of bacto tryptone (BD,Ref# 221705), 1.25 g of bacto yeast extract (BD, Ref# 212750) and 2.5 g of NaCl (EMD, Lot# sx0402-1) into distilled water for a final volume of 250 ml. The media was then autoclaved for 15 minutes (8).

***E. coli* strain and growth.** A total of 4 *E. coli* strains were obtained from Trout Lake samples, with two of each duck and gull species. The strains were designated as TrGu 95-1, TrGu95-2, TrDu 95-1 and TrDu 95-2. The frozen isolates were first thawed from -80°C and then streak onto MacConkey agar plates, which were incubated at 37°C overnight. Red isolated colonies were then selected, inoculated into 5 ml LB starter broth and incubated at 37°C overnight with shaking. A 1/30 dilution of the starter broth was prepared with a final culture volume of 5 ml and incubated at 37°C, overnight with shaking (6).

Cell washing for template. Template preparation was modified from the method described by Zhu (12). Once the *E. coli* culture reached approximately an OD_{600} between 0.6 and 1.2 (Ultraspec 3000, Pharmacia Biotech) 1.5 ml was centrifuged at 13200 rpm for 5 minutes. Then the supernatant was removed and the cell pellet was washed twice with 0.5 ml of 1 M NaCl, followed by 0.5 ml of sterile water. Finally, the pellet was resuspended in 50 ul of sterile water. 1/50 and 1/100 dilutions of the templates were done using sterile distilled water (12).

PCR conditions. For each 50 ul PCR reaction, it contained 5 ul of 10X PCR buffer without $MgCl_2$ (Invitrogen, P/N y02028), 1.5 ul of 50 mM MgCl (Invitrogen, P/N y02016), 1 ul of 10 mM premixed dNTPs (Bio-Rad, cat. 170-8874 and Invitrogen cat.18427-013), 5 ul of 20 uM BOX A1R primer (5'-CTACGGCAAGGCGACGCTGACG-3') (IDT, Lot# 80705802), 0.2 ul of Taq Polymerase (Invitrogen, cat. 18088-042) and sterile water was added to have a final concentration of 50 ul (9). PCR conditions of 94°C for 12 minutes, followed by 35 cycles of 94°C for 1 minute, 55°C for 1 minute and 72°C for 1.5 minutes and ending with a final cycle of 72°C for 7 minutes was performed. For each PCR, a master mix control was run with the samples. PCR for the different templates and different dilutions of the template were done. In addition, different concentrations of 10X BSA, made from 100X BSA (Invitrogen, cat. 18427-029) were added to the PCR master mix to obtain a BSA gradient from 0 to 3.5X BSA of sample. A temperature gradient from 42°C to 64°C with intervals of 2°C was also done to find the optimal annealing temperature. It was found to be 53°C, of which was used in subsequent reactions. The PCR reactions were performed using Biometra T-gradient PCR Thermocycler.

Gel electrophoresis. PCR products were run on 1% agarose gel (3:1 Amresco, Biotech grade, E776-250G, CAS # 9012-36-6) at 90 V for approximately 1 to 2 hours. Gels were made and ran in premade 1X TBE buffer. Each 5 ul sample was loaded with 1 ul of 6X DNA loading dye (Fermentas, Cat. R0611). In addition, 2 ul of GeneRuler™ 1 Kb DNA bp ladder (Fermentas, Cat. R0611) made up to 5 uL with distilled water was loaded with each gel. The gels were then stained in 0.5 ug/ml ethidium bromide for 1-2 hours. The gels were imaged using UV Gel Doc (Multimage™ Light Cabinet).

RESULTS

Template type, amount and concentration. The two different *E.coli* strains of duck and gull were tested for the effect of different amounts of template in the PCR and different concentrations on the templates (data not shown). Templates of 0.25, 0.5, 1.0 and 1.50 ul of TruGu 95-1 and TruDu 95-1 were added to 25 uL PCR reactions containing BOX A1R primers. A master mix and negative control were loaded along with the samples. Bands were the most resolved at 1.0 and 1.5 ul for both gull and duck samples as it appeared that the addition of more template yielded brighter and more reproducible bands by 2 times. Subsequent PCR reactions used 2 ul of the template for templates with dilutions. After the cell wash, dilutions of 1/50 and 1/100 of the 50 ul template was done using distilled water. Then 2 ul of the template was loaded with 25 ul PCR reaction along with a master mix and negative control. It was found that templates with a 1/100 dilution gave brighter and more visible bands compared to templates with no dilution and a 1/50 dilution.

Annealing temperature and reproducibility of PCR between species. Following, a temperature gradient of TruGu 95-2 with a 1/100 dilution was done. This was to test for the reproducibility of the band and to find an optimal annealing temperature for the BOX A1R primers (Fig. 1). The temperature gradient was from 44 to 62°C in increments of 2°C. From the resolved gel, it was observed that low annealing temperatures produced clearer bands that had higher molecular weights (500 to 1000 bp). However, although at high temperatures, the bands with high molecular weights were not well resolved compared to the bands

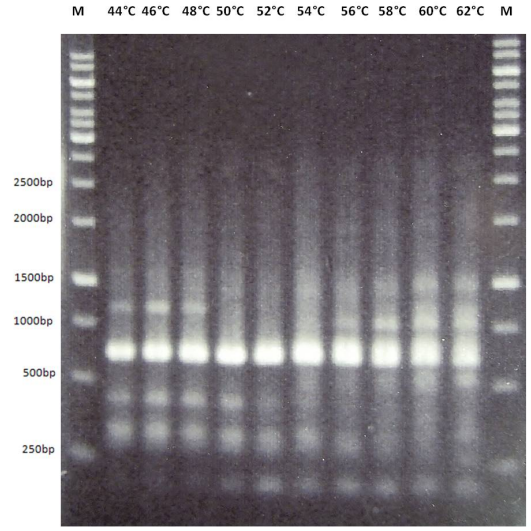

FIG. 2. Temperature effect on PCR products from washed 1/100 dilution *E. coli* isolates of TrDu95-1. The values alone the top of each lane represent the temperature gradient used, with 2°C increment increase from right to left, from 44°C to 62°C. The molecular weight makers identified as M are GeneRuler™ 1Kb ladder from Fermentas.

produced at low annealing temperature, where larger number of low molecular weight bands appeared around 250 to 500 bp. It was observed that using an annealing temperature of 53°C gave the best clarity of high molecular weight bands while maintaining the presence of low molecular weight bands. It also produced the most visible bands.

To investigate the reproducibility of the PCR conditions of *E. coli* sources from different species, the same PCR conditions such as reagent amounts and PCR cycles lengths and times for gull samples were used in duck isolates (TruDu 95-1) (Fig. 2). A 1/100 dilution was also done for the template after cell wash preparation. Then a temperature gradient PCR from 44 to 62°C in increments of 2°C was performed. Band patterns for duck sources were observed to be similar to the patterns from gull sources. High annealing temperature produced more resolved low molecular weight bands (250 to 500 bp) while high molecular weight bands (500 to 1000 bp) were more visible for low annealing temperature. In addition, the optimal annealing temperature for *E. coli* from duck sources was still observed to be 53°C. Thus an annealing temperature of 53°C was chosen for subsequent experiments. Finally, it appears that different annealing temperature also produced different visible bands and also bands with different intensity.

Template age and addition of BSA. In order to investigate the effect of cell age on PCR reactions, cells in different growing stages were used. The different turbidity levels represented different growing phases of the cells. An OD level less than 1.2 was assumed to correspond to the exponential growing phase while an OD level past 1.2 was assumed to correspond to the stationary phase (1, 11, 12). This was to test the effect of the growth stage in PCR reactions. Two samples of gull and duck (TruGu 95-1 and TruDu 95-1) were grown to two different OD levels, one in exponential and another in stationary phase. For this gel, sample TruDu 95-1 with OD of 1.097 was the only sample of the four to have no bands apparent (Fig. 3).

In addition, different concentrations of BSA in the PCR reaction were tested. Different amounts of 10X BSA were added into the PCR reaction. PCR reactions with no BSA to 3.5X BSA were tested (0 ul to 7 ul of 10X BSA) (4). It appears that the addition of 10X BSA actually decreased the intensity of the PCR bands by 2 times (Fig. 3).

DISCUSSION

As a starting point in PCR optimization, literature conditions using cell washing for template were duplicated (12). As mentioned in the results, 1/100 dilution of both duck and gull templates was the most optimal concentration. This dilution consistently produced the brightest and most visible PCR products when compared to zero dilution and 1/50 dilution. In this experiment, the templates were prepared by whole cell lysis using cell wash method (12). Since the washed cell method did not necessarily remove all of the cellular debris after centrifuge, the final template samples might have contained a small amount of cellular debris mixture that might have dampened the efficacy of PCR. In 1/100 dilution sample, the amount of cellular debris present was significantly less than those in samples of 1/50 and no dilutions, which would mean less interference for the PCR reaction. In addition to cellular debris, the ions from the NaCl wash could have interfered with the function of *Taq* polymerase (12). As less NaCl was present at lower concentration of template solution, the *Taq* polymerase would have been subjected to lower interference and would have performed at a higher efficiency, thus allowing higher yield of PCR products.

The literature value of PCR conditions of 94°C for 2 minutes, 32 cycles of 94°C for 1 minute, 55°C for 1 minute, 72°C for 1.5 minutes and a final cycle of 72°C for 7 minutes was investigated. In addition, the usage of 1 ul of 25 mM dNTPs and 25 ul of 20 uM BOX A1R

primer were also tested (12). The bands produced were faint and irreproducible. Thus PCR conditions of 95°C for 2 minutes, and 32 cycles of 94°C for 3 seconds, 92°C for 30 seconds, 50°C for 1 minute and a final cycle of 68°C for 8 minutes were investigated (2, 6). However, this produced no bands. Thus, PCR conditions from the first trial was modified to 94°C for 10 minutes, 32 cycles of 94°C for 1 minute, 55°C for 1 minute, 72°C for 1.5 minutes and a final cycle of 72°C for 7 minutes. In addition, dNTP concentrations were changed to 1 ul of 10 mM premixed dNTPs and the primer concentration was increased to 5 ul of 20 uM BOX A1R primer (9). Prolonging the time at 95°C from 2 to 10 minutes might have helped to break open the cell walls and release DNA into the PCR reaction, similar to a heat lysis treatment (12). In addition, by increasing the concentration of our dNTPs and primers, it reduced the production of faint bands. Thus, our new PCR conditions produced reproducible bands and eliminated the problem of low intensity bands, solving the problem of reproducibility and low intensity bands produced by BOX A1R PCR (6). This is significant because reproducibility and intensity of BOX A1R PCR products are of high importance in the application of MST for fecal contamination analysis.

Reproducibility between different species of *E. coli* isolates was also tested through a temperature gradient to deduce whether BOX AIR PCR was reproducible for different species and if it could produce characteristic bands for different species. As seen in Figures 1 and 2, BOX AIR PCR was reproducible for duck and gull. In addition, the effect of the annealing temperature gradient was similar in both species, in that low molecular weight bands were more apparent with high annealing temperatures whereas high molecular weight bands were more visible for low annealing temperatures. In addition, for different annealing temperatures, different bands were apparent and had different intensities. It appears that each different species had different bands visible and of different intensity, consistent with literature. However, from Figures 1 and 2, it was concluded that an annealing temperature of 53°C produced the most resolved bands at both high and low molecular weight.

In attempts to obtain even more intense and consistent bands, template cell age and BSA concentration were tested. Investigations by both Abolmaaty *et al.* (1) and Zhu (12) looking at the correlation of culture growth phase and the amount of DNA released from different lysis techniques demonstrated that there were no differences between exponential and stationary generations of culture (1). This observation was consistent with our results as all the bands observed for both phase samples were of similar intensity level (Fig. 3). This could mean that the result of the quantity of DNA released from younger and older generations was similar, or that the number of cell cycles used in this experiment was not optimal for differentiating the difference. Either of these reasons would explain the similarity across all the samples from different growth phases.

One of the exceptions, however, was observed in 1/100 dilution of the TrDu95-2 sample, where no bands were visible in comparison to other samples. This was not in agreement with the previous experiment performed by Zhu (12) or with the previous temperature gradient PCR in the study. The study by Zhu utilized templates that had a turbidity of less than 1.2 OD_{600} and demonstrated well-defined results for the PCR product (12). Our previous temperature gradient PCR also used templates that had an OD_{600} less than 1.2 and performed reproducibly with good intensity (Fig. 1 and 2). Thus this inconsistency was probably due to a potential human error.

The addition of BSA to PCR reactions was also investigated. This is because BSA has been shown to effectively reduce the influence of PCR inhibitors in complex samples such as feces, blood and meat and may increase the reproducibility of band patterns (6). This may be because BSA overcomes PCR inhibition by binding to blood-or heme-containing compounds and phenolics in the samples. Finally, BSA can bind to proteinases found in fecal samples that cause DNA polymerase degradation. In the study by Lee and Wong (6), 400 ng/ul of BSA was shown to have an enhancing effect on the reproducibility and intensity of the PCR bands. However, since our *E. coli* originated from simple LB broth with no meat, blood or feces present, the addition of BSA had negative effects on our PCR. In addition, the concentrations that were used in this experiment were considerably higher than that used in the study by Lee and Wong, and might have caused the interference in the PCR process.

FUTURE DIRECTIONS

Using nucleotide BLAST analysis of the primers against the entire *E.coli* genome, it was found that many matches of the primers to the genome were possible. However, those matches were only partial matches and only some of the matches contained the 3'OH end of the primers. Thus, more studies in the future is needed to evaluate whether there are more compatible primer designs and M-fold analysis is needed to provide better alignment to the template and non-folding secondary structures. This optimization would increase the

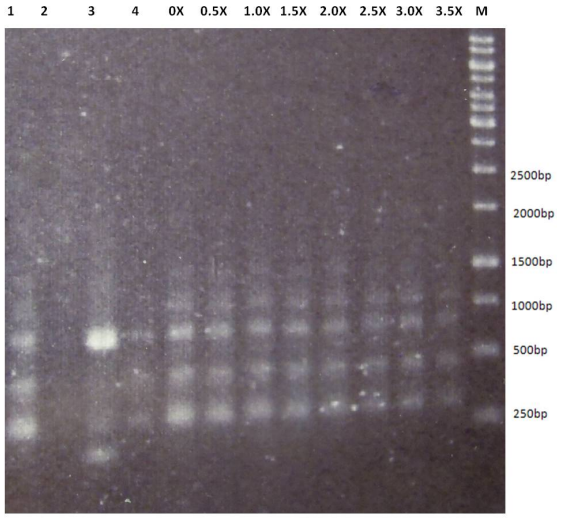

FIG. 3. The effect of template age and BSA concentration on the PCR products from *E. coli* isolates of both TrDu 95-1 and TrGu 95-1. Lanes labelled 1 to 4 represent: (1) TrGu 95-1 with OD_{600} of 1.030 and (4) OD_{600} of 1.435, and (2) TrDu 95-1 with OD_{600} of 1.097 and (3) OD_{600} of 1.414. Lanes containing samples with different concentrations of BSA are labelled, with their corresponding concentrations along the top of each lane.

reproducibility of the PCR reactions because the primers would have more reproducible alignment to the template.

In addition, only the intensity and reproducibility of the bands were investigated in this experiment. Improvement of band resolution can be improved by investigating the type of agarose used, the percentage of agarose used in the gel, the voltage and also the temperature of the gel. Lee and Wong (6) showed that agarose gels left to solidify in the fridge gave better shaped bands. Combining better resolution with our PCR conditions would allow the study of more *E. coli* isolates can be investigated to determine if they produced sufficient bands with different intensities to be suitable for MST analysis to identify different sources of *E. coli* contamination (5, 7).

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