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Testing the Efficacy of PCR for DNA Fingerprinting Avian Isolates of *E. coli* from Trout Lake using the *rpoS* Gene

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The DNA fingerprinting technique using the *rpoS* gene product was investigated. DNA fingerprints for various samples of *E. coli* isolated from gull, duck and unknown sources were generated by creating customized primers for two distinct but adjoining regions within the *rpoS* gene to study the quantitative presence of the gene within different hosts by PCR. Optimization needed to be done at numerous steps of the PCR protocol, involving variables such as dNTP concentration, dilutions, duration of steps and annealing temperatures, as well as other procedural details such as centrifugation and agarose content in gels. Though the results improved progressively after incorporating various changes in protocol, a lot more optimization needs to be done before results can be studied quantitatively to differentiate between gene expression in various host-specific strains.

The exponential growth of human population in North America is reflected by an increase in residential, commercial as well as industrial development. Because of such widespread growth, there is a great amount of waste produced (3). Sometimes, due to waste-managing accidents or malfunctioning infrastructure, sewer overflows may cause serious contamination within groundwater and other water bodies. In such cases, it is important to determine the source of fecal contamination in order to curtail the incidents and potentially prevent a re-occurrence. Microbial Source Tracking (MST), a system that identifies the source of contamination based on unique microbial identifiers, has been suggested as a means of monitoring contamination (5).

Lee and Wong (5) suggested using repetitive element sequence-based PCR (rep-PCR), using the BOX-A1R primer for the purpose of DNA fingerprinting in various *Escherichia coli* strains. However, their results showed many inconsistencies. After trying out various methods to optimize their experiments, they suggested numerous ways to improve the analysis using their original method (5). There is a chance that their original approach may not necessarily be compatible, since their original consensus sequence for their specific genes were initially obtained from *Streptococcus pneumoniae.* They also mentioned the presence of limitations due to geographic and temporal limits of the gene library (5).

Instead of using BOX-A1R, one could still use PCR fingerprinting techniques to identify host specific strains of *E. coli* by using the *rpoS* gene as a MST tag. This gene transcribes a sigma factor, σ^{38} , which regulates stationary inducible genes in stressful conditions (4,9,10). Since the expression of this gene depends on the type of nutritional and other environmental limitations, and since the internal environment is different from host to host, the differential expression of this gene can be used to potentially identify host-specific *E. coli*. PCR can be used to amplify copies of the *rpoS* gene and then subsequent gel electrophoresis data can be used to form a correlation between gene expression and host-specific strains. For this reason, this project aims to investigate the feasibility of using the rpoS gene as a potential MST candidate in identifying host specific strains of *E. coli*

METHODS AND MATERIALS

***E. coli* sources and preparation**. *E. coli* isolates used in this set of experiments were obtained from the Trout Lake Bird Samples, stored at -80°C freezer in the Wesbrook Building. Samples were chosen randomly (Table 1), thawed in water at room temperature and then tested for confirming identity; subjecting subcultured samples to a catalase test and an oxidase test to determine negative results was sufficient. Confirmed *E. coli* isolates were then grown in Luria-Bertani broth; 10 g tryptone (Control #750440, DIFCO), 5 g yeast extract (Lot # 7116730, BD Bioscience), 10 g sodium chloride (Lot # 055503, Fisher Scientific), made up to 1.0 L, pH 7.4. These isolates were incubated at 37°C and grown for approximately 18-20 hours, after which turbidity was measured to ensure they had reached the stationary phase. 1.0 ml samples of the same were measured using a spectrophotometer to see if OD_{660} was above the range of 0.5 – 0.7. After confirming optical density, 1.0 ml of each sample was washed by centrifuging and removing supernatant, and then resuspended in 1.0 ml dH_2O. Four subsequent ten-fold serial dilutions were then made from each washed sample. All diluted samples were appropriately labelled and used for a variety of suspension PCR tests.

PCR reagents / primer preparation: All primers were obtained

TABLE 1: Trout Lake avian *E. coli* isolates used in this study

Identification Code	Host	Experimental Label
A5-82	Duck	D1
A5-85	Duck	D2
A5-23	Gull	G1
S10-5	Gull	G2
S10-7	Gull	G3
S10-12	Gull	G4
95-1	Unknown	U1
95-6	Unknown	U2
95-21	Unknown	U3

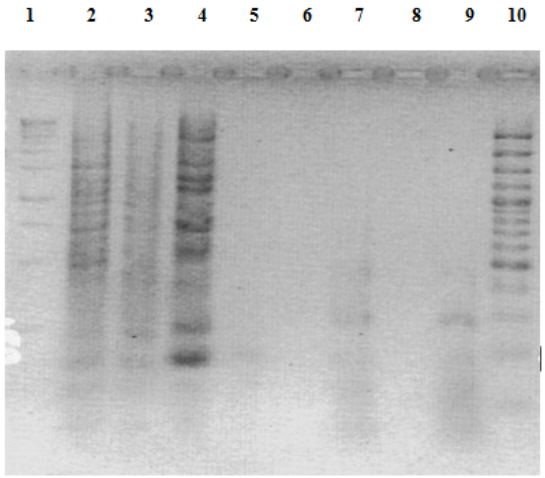

FIGURE 1: The effect of increased annealing temperature on the PCR. After much more optimization, the gel here shows much better resolution with more distinct bands. The 1 Kb ladder is represented in lane 1, and the 100 bp ladder is in lane 10. Lanes 2 – 4 represent samples G2, D1, D2 respectively with primers 1+2, and lanes 7 – 9 represent the same samples with primers 3+4. Lane 6 represents the negative control. A higher annealing temperature of 55°C shows the best results for primers 1+2, with the brightest bands seen in the desired region, representing the gene product of 167 bp. Results are inconclusive for primers 3+4.

in lyophilized form, to which distilled, autoclaved water was added such that final concentration for each primer stock solution was 100 µM (Primer 1, GAGATGTCGCCTCTCGCCGC; Primer 2, GCGGAAACCACGTTCCGGGT; Primer 3, ACCCGGAACGTG GTTTCCGC; Primer 4, CCCAGCGGGGTGTCTACCGA). For instance, 343 µL of d H_2O were added to the tube containing 34.3 nanomoles of the primer. Other PCR reagents used were already at their stipulated concentrations. Three different PCR kits were used (Invitrogen, Fermentas, BIO-RAD) owing to various problems encountered during the experiment.

PCR conditions and parameters: The proportions of each ingredient used for all PCR runs were kept constant. For each run, the master mix was made first (2.5 µl 10X PCR buffer, 0.625 µl 50 mM $MgCl_2$, 2.5 µl 2mM dNTPs, 17 µl dH_2O, 0.25 µl whole cell suspension) excluding the Taq polymerase and the primers, heated at 95°C for 5 minutes to lyse all the bacterial cells in the suspension, centrifuged at 7000 rpm for 30 seconds, after which Taq polymerase (0.25 µl of 5 U/µl) and primers (0.125 µl each) were added. A washed blank sample was used in place of whole-cell suspensions as a negative control for each run. Additionally, for each run, only one set of primers was used at a time – either a set of primers 1+2 or a set of primers 3+4 were used for an individual trial.

All samples including the negative controls were subjected to PCR initially using the regular thermocycler (initial denaturation 95°C for 5 min; 35 cycles of 30 s at 95°C, 30 s at 45°C, 60 s at 70°C; final elongation of 10 min at 70°C), and later using a gradient PCR thermocycler (annealing temperatures 49°C - 65°C). The results were studied after each PCR run, certain variables were optimized including doubling the denaturation and annealing to 1 min, and the elongation to 2 min and the samples were run again.

Electrophoresis and post-staining: All samples were run initially on a 1.5% agarose (Control # BR45080027, BIO-RAD) gel, later changed to 1.75%. The gel was run in 1X TBE Buffer, made as follows: 1 liter of 10X buffer consisting of 104g Tris Base (Lot # 083736, Fisher Scientific), 54 g Boric Acid (Lot # 110140271, Sigma), 40 ml 0.5 M EDTA solution (500 ml solution made using 93.05 g EDTA disodium salt (Lot # 935918A, Fisher), pH adjusted to 8.0 using 15-20 g sodium hydroxide pellets (Lot # 880519, Fisher), topped to 500ml with dH_2O), topped to 1.0 L with dH_2O. Two ladders were used along with each set of PCR runs, both having a final concentration of 0.5 µg of DNA; for instance, 9 µL of d H_2O was added to 1 µL of 0.5 µg/ µL of the Gene Ruler 1KB ladder (Lot # 00032587, Fermentas). The other ladder used was Gene Ruler 100bp (Lot # 00028313, Fermentas). Gels were loaded with 10 µL of each sample containing an additional 2 µL 6X GLB dye (Lot # 00034553, Fermentas) and electrophoresed at 90 V until dye fronts had moved to required point. After electrophoresis, gels were stained in tub containing 350 mL 0.5 µg/ml ethidium bromide solution for 15-20 minutes. The gels were then visualized under Trans-UV and Epi-White lights, using AlphaImager software.

RESULTS

To conduct a PCR analysis, suitable primers needed to be designed. In doing so, one has to determine the linear DNA folding regions to be avoided, to enable proper annealing of the primers during PCR. For this purpose, the *rpoS* gene sequence (993 bp) was obtained from EcoGene. A BLAST analysis revealed up to 50 matches with 100% similarity and gene identity ranging from 94-100%. Other notable information obtained from the matching revealed that many sequences had a single nucleotide polymorphism at the 100^{th} and 790^{th} base pairs. This gene sequence was subjected to a M-fold analysis (37 C, [Na+] = 1.00, [Mg2+] = 0.00). Based on the data obtained, the gene sequence was run through NCBI's Primer design to find a suitable pair of primers that conform to the conserved region of the gene, avoiding the polymorphisms. Two different sets of primers, selected to represent different but adjacent portions within the conserved region, were thus developed (Lot # 553881, Reference #s 48208652 – 54, Integrated DNA Technologies) and used for all subsequent experiments.

The results given here follow the progression of changes mentioned to optimize amplification of *rpoS*. The first few gels visualized after running PCR yielded

no results, indicating that the reaction wasn't successful. Upon increasing dNTP concentration, some PCR products were seen, but not very clearly. The fuzziness was deduced to have been caused by cellular debris and other particulate matter suspended within the PCR mixture. After proper centrifugation, it was slightly easier to see some genetic material present in each lane, but the resolution of the gel was still rather unclear. Increasing the amount of agarose in the gel, along with appropriate heating and cooling of the gel mixture prior to pouring led to the creation of clearer gels, but it was still hard to view each band distinctly.

After making a few other minor changes, it was seen that the Invitrogen PCR kit stopped functioning properly. Switching over to a Fermentas kit also produced no results. However, results were obtained when a BIO-RAD kit was used. To see if the results previously obtained were still reproducible with a different Invitrogen kit, the same PCR conditions were maintained and samples were run as usual, but again, for some reason, nothing was being visualized, even after replenishing the ethidium bromide staining solution. As a result, all subsequent trials were run with BIO-RAD reagents. Furthermore, the duration of each step within the PCR cycle was doubled, to ensure a more complete reaction, thus increasing the amount of PCR product. Significantly improved results were observed. However, it was still hard to distinguish between bands. It was predicted that the whole cell suspensions used were far too concentrated, and thus a series of different dilutions were also run under uniform PCR conditions. Since the best results were seen with a 1/10 dilution, all subsequent trials were conducted using 1/10 dilutions for all sample suspensions.

Since the PCR reaction is very sensitive to changes in temperature, especially considering how different sets of primers and their PCR products have different melting points, identifying the ideal annealing temperatures was the next most important step. Increasing the annealing temperature from 45°C to 55°C showed significantly improved results (Fig 1). As a result, a temperature gradient PCR was run using a single sample but with both sets of primers, the results from which confirmed the notion that the optimum temperature of annealing for both PCR products varies greatly (Fig 2).

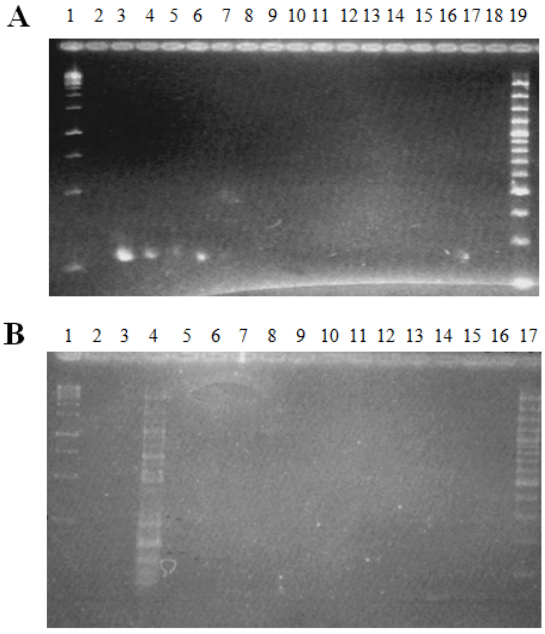

FIGURE 2. Effects of using a temperature gradient on the annealing temperature of the PCR. Sample D2 is represented in (A) and sample G3 is represented in (B). For both, the 1 Kb ladder is in lane 1 and the 100 bp standard is in lane 18. The temperatures (in °C) 49.0, 51.4, 53.8, 56.2, 58.6, 60.9, 63.1, 64.5, 65.0 are represented in lanes 2 – 11 respectively. These images reflect results seen for primers 1+2 only, as trials for primers 3+4 did not yield positive results. As seen in both, the temperature of 58.6°C seems to work best, especially for (B).

DISCUSSION

As the PCR product was not seen in the first few attempts, and since it was seen by significantly increasing the concentration of dNTPs in the PCR mix, it can be concluded that appropriate expression of the gene product requires the presence of sufficient dNTPs. Indeed, since the gene products here are 167 bp and 277 bp, for primers 1+2 and primers 3+4 respectively, a shortage of dNTPs would greatly diminish the scope of amplifying the gene product within PCR. Going from a concentration of 2mM to 25mM is an almost 12-fold increase, but it may be better to have an excess of nucleotides, to maintain a good supply of dNTPs, ensuring that the reaction goes to completion. There is a chance that having a large excess of nucleotides may cause the visualized gel to appear blurry towards the end, owing to the expression of single nucleotides, but considering that this region is far away from the region of interest, namely between 100 and 300 bp, the presence of small, blurry bands towards the very end would be inconsequential. However, research has shown that an extreme excess of dNTPS along with excess $MgCl_2$ concentration within the PCR master mix can be detrimental for the reaction (6). Since dNTPs are frozen at -20°C, freezing causes some degradation, and incorporation of degraded dNTPs may damage the PCR product (6). This might have been the case with the Invitrogen kit, which started malfunctioning after working initially. The failure of the Fermentas kit may also be attributed to degraded dNTPs and/or other reagents within the kit. Additionally, since Mg^{2+} ions form complexes with dNTPs during the reaction, the concentration of $MgCl_2$ has to be at par as well (6). A series of optimization tests using varying concentrations

dNTPs and $MgCl_2$ must be carried out before finalizing concentrations for subsequent experiments.

An appropriate amount of centrifugation was necessary prior to and subsequent to a PCR reaction. Incorporating more centrifugation yielded clearer visualized bands diminished trails or missing regions. Considering the usage of whole cell suspensions for all the experiments in this study, all the dense cellular debris that remained behind after lysis must have potentially clogged the pores within the gel, resulting in poor banding and thus causing blurring. This was especially important for conducting quantitative studies after visualizing the gel, as blurred / indistinct bands are extremely hard to quantify based on intensity and shape. The effect of cellular debris marring the results was amplified further when the % agarose within gels was increased; as pores became smaller, there was more resistance against the contents within the well to move in the opposite direction, and the pressure exerted by unwanted particulate matter would disrupt the pores, resulting in an uneven distribution of the contents. Complementing the same considerations, it is of utmost importance to use an appropriate dilution of whole cell suspensions, as too high or too low a concentration of genetic material and/or debris may have undesired effects on the efficacy of the PCR reaction. Since DNA is not purified and extracted from its cellular suspension, the latter may contain other degraded nucleic acids and PCR inhibiting factors (6). For this purpose, it is imperative to test out a series of dilutions to determine the dilution that gives the best results. In this case, a dilution of 1/10 seemed to provide the clearest and brightest banding, and hence it was chosen for all subsequent experiments. However, for future studies, one should consider performing DNA extractions to work with purer samples devoid of contaminants and inhibitors.

The duration and temperature of each step within a PCR reaction is critical, especially in the case of annealing. Too low a temperature would lead to formation of non-specific products, whereas too high a temperature might significantly reduce the yield of the final product (2). Generally, the annealing temperature is set 3-5°C less than the stipulated T_m of the primer. In this case, however, using the recommended T_m as the annealing temperature did not yield the best results, so a temperature gradient PCR had to be performed. Considering that there were two PCR products of different lengths, it was necessary to perform a gradient-PCR for both sets of primers. The results showed an optimum annealing temperature of 58.6°C for the first set of primers, which is substantially lower than the given T_m for both primers, thus indicating the need for testing various temperatures instead of choosing one within a short range of the stipulated annealing temperatures. In contrast, nothing could be concluded about the second PCR product. This was not particularly surprising, considering the information provided by the M-fold analysis; the existence of a hairpin loop in the vicinity of the attachment site of the (-) primer might be significantly inhibiting its hybridization to the template DNA.

These results showed that the *rpoS* gene can be used for fingerprinting, but the methodology requires a more optimization before any proper quantitative analysis can be made. The method will need to be optimized further before assessing whether it has the potential to track microbial contamination.

FUTURE EXPERIMENTS

Perform DNA extractions instead of using whole cell suspensions. To minimize inhibition caused by hairpin loops, test destabilizing solvents like DMSO, formamide or betaine (5,8), but they may reduce activity (7). To stabilize Taq polymerase activity test non-ionic detergents like Tween-20 or Triton X-100 (5,7), but these may increase non-specific amplification.

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