

Optimization and the Robustness of BOX A1R PCR for DNA Fingerprinting Using Trout Lake *E. coli* Isolates

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The rep-PCR DNA fingerprint technique using BOX A1R primer was investigated. DNA fingerprints were generated from *Escherichia coli* strains isolated from human, duck, goose, gull, and heron sources. Optimization tools (use of restriction enzymes, BSA, DMSO, and storage buffer) were used in an attempt to generate clear, well-defined DNA fingerprints from the *Escherichia coli* isolates. Initial studies revealed that although there were distinct fingerprint patterns emerging from the use of BOX A1R primer, the bands were faint and difficult to resolve. The use of optimization tools did not significantly improve the quality of the bands or the reproducibility of the method except for BSA. BSA seemed to improve the reproducibility of the method. This failure in optimizing the rep-PCR DNA fingerprinting technique may be due to the formation of primer dimers. Further research is required into this problem.

Fecal contaminations from agricultural operations as well as urban associated pollution are a persistent problem throughout North America (16). This is due to the increase in residential, commercial, and industrial development; use of manure as fertilizers; the persistence of combined sewer overflows; and malfunctioning septic systems. A practical approach for monitoring contamination is Microbial Source Tracking (MST), which utilizes microbial identifiers to indicate fecal source (16).

Repetitive element sequence-based PCR (rep-PCR) specifically amplifies repetitive portions of the bacterial genome (7, 11). The resulting mixture of amplified DNA fragments is resolved in gels, producing a banding profile referred to as a rep-PCR genomic DNA fingerprint (7,11). Rep-PCR using BOX A1R primer targets naturally occurring, multi-copied, conserved, repetitive, DNA sequences present in the genomes of most gram-negative and gram-positive bacteria (20). The idea of using this primer is to amplify specific genomic regions located between BOX elements (154bp) and its natural inverted repeats (2). The BOX elements were originally discovered in *Streptococcus pneumoniae* typically occurring in approximately 25 copies (20). These repeats were found to consist of three discriminate regions: *boxA*, *boxB*, and *boxC*, which are 59, 45, and 50 base pairs in length respectively. Alignment of several *boxA* sequences in *Streptococcus pneumoniae* gave consensus sequence illustrated in figure 1 (2).

Band profiles generated from using BOX A1R primer are powerful for determining and clustering inter and intra-species; as well as being a promising

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1      10      20      30      40      50      59  
cons. TAATACTCTTCGAAAATCTC.TTCAAACCACGTCAGCGTCGCCCTTCCCGTAGATAT.GTTA
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FIG. 1. Consense sequence from multiple alignment of several *boxA* sequences in multiple region of *Streptococcus pneumoniae*: hexB, SII, mrsA, comA, SI and ply (2).

candidate for pathogen profiling and MST. Thus, the banding patterns from a library of *Escherichia coli* isolates from various hosts can serve as “fingerprints” and be ultimately used to predict the host source of unknown *E. coli* samples. BOX A1R PCR studied in the context of *E. coli* microbial source tracking was promising (6,7,11). However, limitations were also demonstrated due to geographic and temporal limits of the library, and non-host specific distribution of bacterial isolates (19). Reproducibility problems were also demonstrated in previous studies (20).

A major concern for using a library-based method is that the accuracy for future assignment of unknown isolates is dependent on the integrity of the band profile. Thus, a set of sharp, unique, and high-quality band patterns is crucial when using BOX A1R PCR for MST. In this experiment, various parameters were studied to assess the robustness of the method and whether the PCR reaction could be better optimized than literature protocol (7).

MATERIALS AND METHODS

***E. coli* sources and verification.** The *E. coli* isolates used were obtained from Trout Lake samples, human clinical sample, and a lab

Table 1. *E. coli* isolates used in the study.

Host	Identification Number	Site
Human	Clinical 241	Clinical
Gull	A523	Trout Lake
Duck	A582	Trout Lake
Goose	951	Trout Lake
Heron	9564	Trout Lake
Lab strain	Strain C29	Micb 421 culture collection

strain (Table 1). After thawing out the isolates that were stored in the -80°C freezer, a series of culturing were done to ensure that the isolates were *E. coli*. Firstly, the isolates were grown out on TSA plates (Lot# 7278163; BBL™ BD, ON, Canada) and then grown on EMB agar (Lot#85727294, Lot#1342; BDH, VWR CANLAB, Mississauga ON, Canada) as well as Gram stained. The tests indicated that the isolates were gram all negative rods that were positive for lactose uptake.

***E. coli* Preparation and PCR Conditions.** *E. coli* was prepared and PCR was performed in 3 different conditions. In all three conditions, the primer, BOX AIR primer (5'-CTACGGC AAGGCGACGCTGACG -3'), being used was the same. The 100 µM BOX AIR primer solution (Lot# 81207406, Ref#39610986; IDT, Toronto ON, Canada) was made by suspending 107.20 nmoles of the primer in 1072 µl sterile water (Lot#605703 Cat# 15230170; Invitrogen, Burlington ON, Canada). The stock primer solution was then diluted 5-fold to get a 20 µM solution which was used in subsequent PCR reactions. Also, the temperature cycles were kept the same in all the different PCR runs. PCR was initiated by incubating the reaction mixture at 95°C for 2 minutes, followed by varying number of cycles consisting of 94°C for 3s, 92°C for 30s, 50°C for 1 min, and ending at 65°C for 8 min. The PCR reactions were performed using a Biometra T-gradient PCR Thermocycler (Montreal Biotech Inc, Kirkland P.Q., Canada).

Colony PCR Reaction. *E. coli* isolates were grown for approximately 18 hours on TSA plate and then washed and re-suspended in 100 µl sterile water followed by a heat lysis step for 10 minutes at 95°C producing a whole-cell suspension. Table 2 is the original master-mix according to literature (7,11). Three reactions (1A, 1B and 1C) were set up for colony PCR. (Table 3).

Suspension PCR. The *E. coli* isolates were grown for approximately 18 hours in Luria-Bertani liquid medium (10.0 g tryptone, 5.0 g Bacto™ yeast extract(Lot#7116730, Ref#212750; BD Bioscience, Mississauga ON, Canada), 10.0 g sodium chloride(Lot#055503, Cat#S271-3; Fisher Scientific, Ottawa ON, Canada), made up to 1000mL at pH 7.5 – 8.0). 500 µl of the LB broth was then spun down and washed with sterile distilled water once. The pellet was then re-suspended in 100 µl of sterile distilled water followed by a heat lysis step (heating for 10 minutes at 95°C). Seven cell suspension PCR were performed (2A, 2B, 2C, 2D, 2E, 2F and 2G) with 35 PCR cycles per reaction. Various isolates were used in the negative control reaction for 2A to 2F but sterile distilled water was used in place of primer and Taq polymerase for the negative control reactions. Table 4 provides the different sets of PCR performed using cell suspensions.

Restriction Enzyme. Two types of restriction enzymes were used in this series of PCR reactions. XbaI (Lot#2502116; Cat#15226-012; Invitrogen, Burlington ON, Canada) and NotI (Cat#15441-017; Invitrogen, Burlington ON, Canada) cleavage sites were examined prior to usage to ensure that the BOX primer would not be cleaved. The isolates were first grown in LB broth for approximately 18 hours and 1000 µl of each isolate was then washed and spun down in distilled water once and concentrated into 500 µl of sterile distilled water. They were then heat lysed at 95 °C for 10 minutes and slowly cooled to 30 °C at a rate of 1 °C/minute. After lysis, the DNA of each

Table 2. Master-Mix of PCR Reaction.

Reagent	Amount (µl)
Whole cell suspension	2.00
10X Buffer (Lot#1339626, P/N Y02028; Invitrogen, Burlington ON, Canada)	2.50
25 mM dNTP's (Part# 55082 – 55085; Invitrogen, Burlington ON, Canada))	1.00
50 mM MgCl (Lot#477113, P/N Y02016; Invitrogen, Burlington ON, Canada))	1.50
20 µM BOX AIR primer (Lot#81207406, Cat#39610986; IDT, Toronto ON, Canada)	2.50
Taq Polymerase (Lot#486001, Cat#18038-042; Invitrogen, Burlington ON, Canada))	0.20
Sterile Water	15.30
Total Volume	25.00

Table 3. Sources of isolates used in specific reactions.

Reaction	PCR cycles	Isolates
1A	30 PCR cycles	Goose, human, heron, gull and duck Negative control: distilled water
1B	35 PCR cycles	Goose, human, heron, gull and duck Negative control: distilled water
1C	30 PCR cycles followed by re-amplification with 35 PCR cycles	Goose, human, heron, gull and duck Negative control: distilled water

isolate was measured using the Biochrom Ultrospec 3000 UV/Vis spectrophotometer (Amersham Pharmacia Biotech, GE Healthcare Life Science, Baie d'Urfé Québec, Canada) and the isolates were digested overnight at 37 °C with a final concentration of 0.5 Units of enzyme per µg of DNA. The digestions were terminated by heating the digests at 65 °C for 10 minutes. Seven PCR reactions using 35 PCR cycles (unless stated otherwise) were performed on the restriction enzyme digested samples (reactions 3A, 3B, 3C, 3D, 3E, 3F and 3G). Two-fold increase in Taq polymerase (0.4 µl) was used for reaction 3B to 3G. Water volume was adjusted to a final volume of 25 µl per reaction. Negative controls were used in all reactions where water was used in place of Taq polymerase and BOX primer for a digested isolate. Undigested samples that were heat lysed and incubated without restriction enzyme were also used and a positive control where the DNA was heat lysed only and not submitted to restriction digests. Table 5 illustrates each reaction.

For reactions 3D to 3G, two potential optimization reagents were used to sharpen the band in the genomic DNA fingerprint. The optimization reagents used were purified Bovine Serum Albumin (BSA) 100X (Lot#0405, Cat#B90015; Fermentas BioLabs New England, Burlington ON, Canada), and storage buffer (4 µl 25 mM EDTA (Lot#00013410, Cat#R1021; Fermentas, Burlington ON, Canada), 100µl Tris-HCl buffer, 500 µl glycerol(Lot#38182849, Cat#B28454-76; BDH, VWR CANLAB, Mississauga ON, Canada), 1.543µg of dithiothreitol Cleland's Reagent Electrophoresis Purity Reagent (Lot#M2654; Cat#161-0611; Biorad, Mississauga ON, Canada) and 386µl sterile distilled water). Storage buffer ingredients was based from storage buffer for DNA polymerase as stated by manufacturer (Lot#486001, Cat#18038-042; Invitrogen, Burlington ON, Canada). Table 6 provides master-mix preparation for reactions 3D - 3G.

Table 4. Sources of isolates tested with various reaction parameters.

Reaction	Deviations from Table 2	Isolates
2A	None	Goose, human, heron, gull and duck Negative control: duck
2B	100-fold dilution of 2A products followed by another PCR reaction	Goose, human, heron, gull and duck Negative control: duck
2C	Addition of 1.50µl DMSO (adjust water volume to total volume of 25.00µl)	Goose, human, heron, gull and duck Negative control: Gull
2D	100-fold dilution of products from 2C followed by another PCR reaction	Goose, human, heron, gull and duck Negative control: Gull
2E	10-fold dilution of template; 10µl of the cell suspension in LB broth was suspended in 100µl of sterile water	Gull, human and gull cell colony Negative control: lab strain Positive control : lab strain
2F	10-fold dilution of template; 10µl of the cell suspension in LB broth was suspended in 100µl of sterile water	Gull, duck, heron, human and goose Negative control: goose
2G	10-fold dilution of template; 10µl of the cell suspension in LB broth was suspended in 100µl of sterile water	Gull, duck, heron, human and goose Negative control: goose

Gel Electrophoresis. After each PCR reaction, the products were separated on a 1.5% horizontal agarose gel using Invitrogen agarose (Lot # 1137923 Cat # 15510027; Burlington ON, Canada) for all sets of PCR. For 2F, the PCR products were separated on 3 different types of agarose to find out whether the agarose itself could help sharpen the banding pattern. The three types of agarose used were Invitrogen agarose (Lot # 1137923 Cat # 15510027), Biorad Cert™ Molecular Biology agarose (Cat #151-3101; Mississauga ON, Canada) and Amresco agarose 3:1 Biotechnology Grade (Lot# 2006B010, CAS# 9012-36-6; Solon, Ohio). They were all ran in 1X TBE Buffer (10.8 g Tris Base (Lot#1382726, Cat#15504-020; Invitrogen, Burlington ON, Canada), 5.5 g boric acid (Lot#43024326, Cat# BX0865-1; EM Science, VWR CANLAB, Mississauga ON, Canada), 0.74 g EDTA (Lot#1382726, Cat# EX0539-1; EMD Bioscience, VWR CANLAB, Mississauga ON, Canada)). 5-25 µl of each reaction mixture was loaded onto the gel with 1 µl of 6X loading buffer (25 mg xylene cyanol (Lot# 41k3689; Sigma Aldrich, Oakville ON, Canada), 24 mg bromophenol blue (Lot# 962798 Cat# B-397; Fisher Scientific, Ottawa ON, Canada), 3 mL glycerol (Lot# 41087245 Cas# 56-81-5; EM Science, VWR CANLAB, Mississauga ON, Canada) and made to 10 mL with distilled sterile water). GeneRuler™ 100 bp ladder Plus (0.25 – 2.5 µg/well; Lot#00028313 Cat# SM0321; Fermentas, Burlington ON, Canada) was used for measuring the band sizes of the DNA fingerprints and was loaded into each gel in the terminal well as well as the middle of gel or 1/3 and 2/3 way across the gel. The gels were separated at room temperature for 3 hours at 40 V and stained from 15 minutes to overnight with a solution containing 0.5µg to 1.0 µg of ethidium bromide (EtBr) per ml. The gels were then viewed using the Alpha Imager (Alpha Innotech, Fisher Scientific, Ottawa ON, Canada)

RESULTS

Colony PCR reaction. Colony PCR analysis using but faint band patterns among the isolates used (FIG. 2).

Table 5. PCRs performed using restriction digests.

Reaction	Sample preparation	Master mix deviation from Table 2	Isolates
3A	Digests were diluted 10 times		Xba1 Digested goose, gull, duck, and human and a negative control; NotI digested duck isolate and a negative control and undigested sample
3B	20-fold dilution of template instead of 10-fold dilution		Xba1 Digested goose, gull, duck, and human and a negative control; NotI digested duck isolate and a negative control and undigested sample
3C	Dilution series of DNA template at 20-fold, 40-fold and 80-fold		Xba1 Digested goose, gull, duck, and human and a negative control NotI digested duck isolate and a negative control
3D	20-fold dilution of template	Five conditions: 1. BSA (10 µl) 2. BSA (1 µl) 3. Storage buffer (0.2 µl) 4. Storage buffer (0.4 µl) 5. Control (no BSA or Storage buffer) Volume of water adjusted get final volume of 25.00µl	Each conditions: Undigested gull and Xba1 digested duck isolates Negative control using Xba 1 digested duck isolate
3E	Same as 3D	Same as 3D	Each conditions: Undigested gull and Xba1 digested duck isolates Negative control using Xba 1 digested duck isolate
3F	Same as 3D	Same as 3D Lid heating option turned off	Each conditions: Undigested gull and Xba1 digested duck isolates Negative control using Xba 1 digested duck isolate
3G	Same as 3D	Same as 3D with 40 PCR cycles Lid heating option turned off	Each conditions: Undigested human and Xba1 digested duck isolates Negative control using Xba 1 digested duck isolate

Table 6: Master Mixes for Reactions 3D-3F.

Reagent	Volumes (µl) used in different reaction conditions				
	Control	10 µl BSA	1 µl BSA	0.2 µl Storage Buffer	0.40 µl Storage Buffer
1/20 digest	2.0	2.0	2.0	2.0	2.0
10X Buffer	2.5	2.5	2.5	2.5	2.5
25 mM dNTP's	1.0	1.0	1.0	1.0	1.0
50 mM MgCl	1.5	1.5	1.5	1.5	1.5
20 µM BOX A1R	2.5	2.5	2.5	2.5	2.5
Taq Polymerase	0.4	0.4	0.4	0.4	0.4
Sterile distilled water	15.1	5.1	14.1	14.9	14.7
BSA (10 mg/mL)	0.0	10.0	1.0	0.0	0.0
Storage Buffer	0.0	0.0	0.0	0.2	0.4
Total volume	25.0	25.0	25.0	25.0	25.0

Smearing of samples was observed in reaction 1B as the result of an additional reaction of 35 cycles (data not shown). For reaction 1C, although the number of PCR cycles had increased from 30 to 35, no observable bands were seen after exposure in 1.0 µg/mL of EtBr overnight.

Cell Suspension PCR Reaction. Reaction 2A indicated a non-variant heavy band among all isolates

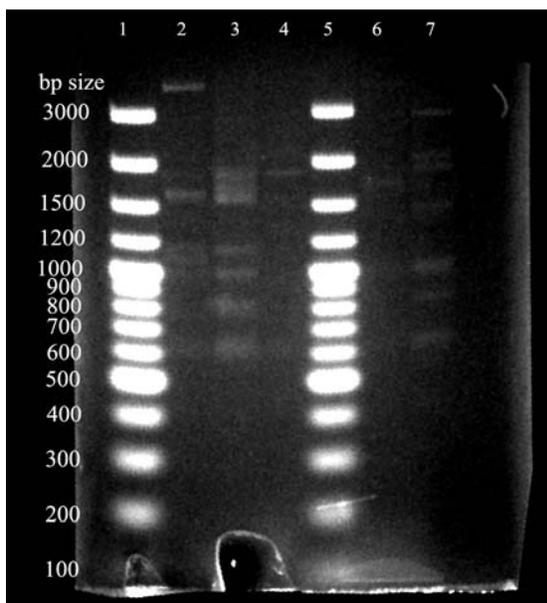


FIG. 2. Colony PCR 1A. Lanes 2,3,4 and 6 contains 5 µl of PCR product of isolates from goose, human, heron, gull and duck isolates respectively; lane 1 and 5 contains 5 µl of the 100bp ladder; lane 7 contains water negative control; gel exposed in 0.5 mg/mL EtBr for 20 minutes.

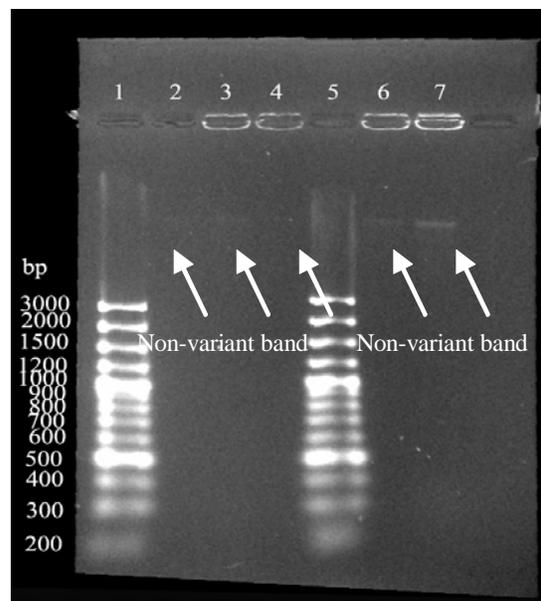


FIG. 3. Cell Suspension PCR 2A. Lane 2, 3, 4 and 6 contains 15 µl of PCR product from of isolates from goose, human, heron, gull and duck isolates respectively; lane 1 and 5 contains 2 µl 100 bp DNA ladder; lane 7 contains duck isolate negative control; gel exposed in 1.0 mg/mL EtBr overnight.

used. The results are shown in figure 3. PCR reactions 2B, 2C, and 2D all resulted in no observable band (data not shown). When decreasing the starting cell material in reaction 2E, multiple faint bands as well as unique banding patterns were observed for each isolate. The result for reaction 2E is shown in figure 4. However, dumb-bell shaped bands were also observed and resolution was still low. Thus, reaction 2F was a confirmation test to ensure that the gel was not the culprit for the oddly-shaped bands and the result for reaction 2F is shown in figure 5. Again, dumb-bell shaped bands were seen in the gel. The PCR products from reaction 2G, with the same conditions as reactions 2E and 2F, were separated in 3 different 1.5% agarose gels made from Biorad, Amresco or Invitrogen agarose but no banding patterns were observed from these products. However, there were also no dumb-bell shaped bands observed in the DNA ladder in the gels, which might be due to prior cooling and solidifying in the fridge overnight before use (data not shown). Therefore, subsequent agarose gels were left to solidify in fridge overnight prior to usage.

Restriction Enzyme. Results from reaction 3A did not yield any bands (data not shown) from the DNA samples digested with XbaI or NotI restriction endonucleases as well as both types of positive control DNA samples. Results from reaction 3B were promising since it was able to yield some faint bands (data not shown). For reaction 3C, twenty-fold and eighty-fold dilutions produced bands for two out of five

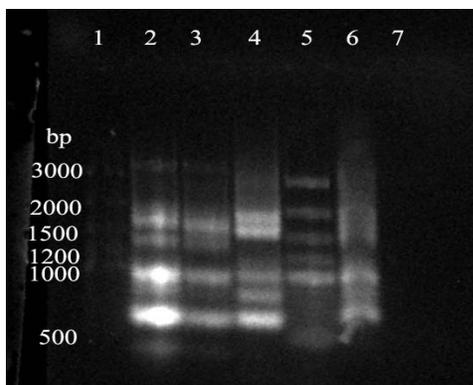


FIG. 4. Cell Suspension PCR 2E. Lane 2 contains 15 μ l of colony PCR product from gull, lane 3 contains diluted cell suspension PCR products of isolates from gull and lane 4 contains diluted cell suspension PCR products from human isolates. Lane 6 contains cell suspension PCR product lab strain *E. coli*; lane 1 and 5 contains 0.5 μ l and 1.0 μ l of the 100 bp DNA ladder respectively; lane 7 contains lab strain negative control; gel exposed in 1.0 mg/mL EtBr overnight.

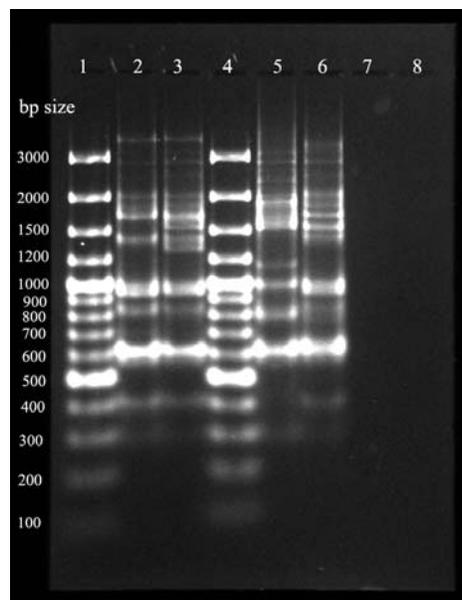


FIG. 5. Cell suspension PCR 2F. Lane 2, 3, 5, 6, 7 contains 15 μ l of PCR products of isolates from gull, duck, heron, human and goose respectively; lane 1 and 4 contains 1 μ l 100 bp DNA ladder; lane 8 contains goose negative control; gel exposed in 1.0 mg/mL EtBr overnight.

samples (duck isolate digested with Xba1 and undigested gull isolate) while the forty-fold dilution produced bands for 1 sample (undigested gull isolate only) (FIG. 6). Because of the promising results in reaction 3C, the amount of Taq polymerase used in the subsequent master mixes were twice the amount of the original amount used.

In reaction 3D, of the five different reaction

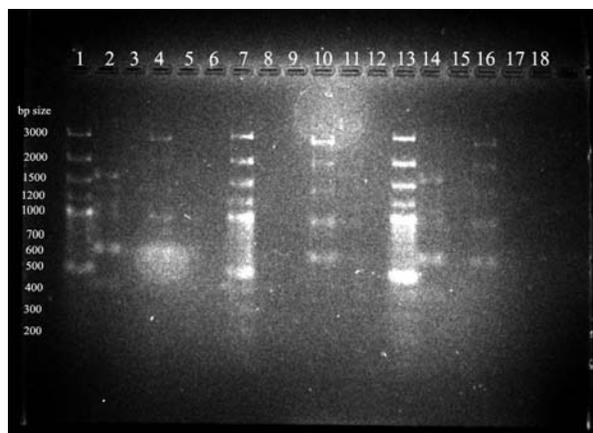


FIG. 6. Restriction digests PCR 3C. Lane 2-6 contains 15 μ l of PCR product for 20-folds dilution material. Lane 8-12 contains 15 μ l of PCR product for 40-folds dilution material. Lane 13-17 contains 15 μ l of PCR product for 80-folds dilution material. Lane 2, 8 and 13 contains PCR product from duck isolate digested with restriction endonuclease Xba1. Lane 3, 9, 14 contains PCR product from goose isolate digested with Xba1. Lane 4, 10, 15 contains PCR product from undigested gull isolate. Lane 5, 11, 16 contains undigested goose isolate. Lane 6, 12, 17 contains PCR product from goose isolate digested with restriction endonuclease Not1. PCR reaction with 2 times original DNA polymerase concentration. lane 1, 7 and 12 contains 1 μ l 100bp DNA ladder; lane 18 contains goose negative control; gel exposed in 1.0 mg/mL EtBr overnight.

conditions, 400 ng/ μ l BSA and control PCR reaction produced banding patterns for one of two of the samples tested (undigested gull isolate DNA only; data not shown).

Duplication of the five different PCR reactions without lid heating (reaction 3E) produced strong bands for undigested gull sample and weaker bands for Xba1 digested duck sample. However, this was limited to 400 ng/ μ l BSA reaction mix only. The results are shown in figure 7.

The remaining products of reaction 3E were separated in a 1.5% agarose gel made with 0.5X TBE buffer and ran in 0.5X TBE. It gave similar band quality (dumbbell shaped bands) as gels using 1.0X TBE buffer (data not shown). It was noted that the heat lid option was turned off 10 minutes into the PCR cycles for reaction 3E. To confirm the effects of turning off heat lid, PCR reactions for reactions 3F and 3G were performed without lid heating from the start. There weren't any bands observed with reaction 3F PCR products (data not shown) and increasing the PCR cycles to 40 in reaction 3G did not increase band signal when compared to reaction 3E. Unlike reaction 3E, 400 ng/ μ l BSA reaction failed to produce any band patterns however, 0.2 μ l DNA polymerase buffer reaction mix yielded band patterns for Xba1 digested isolate. White fuzzy or smeared bands were also observed at the end of the gel for each of the DNA products except for negative controls. The results are shown in figure 8.

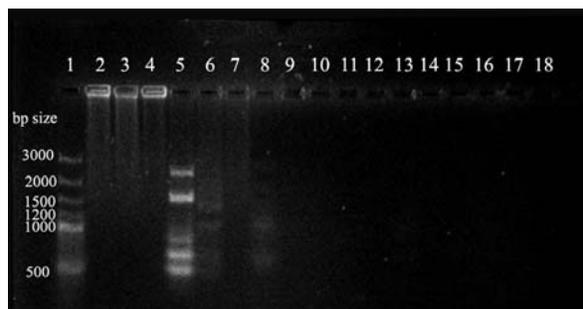


FIG. 7. Restriction Digest PCR 3E. Lane 2-4 4ug/ µl BSA reaction mix; lane 5-7 400 ng/ µl BSA reaction mix; lane 9-11 0.2 µl DNA polymerase buffer/rxn; lane 12,14,15 0.4 µl DNA pol. buffer/rxn; lane 16-18 control PCR mix. Lane 2,5, 9,12,16 contains undigested gull isolate; lane 3,6,10,14,17 contains Xba1 restriction endonuclease digested duck isolate. Lane 4, 7, 11, 15, 18 contains duck negative control. 15 µl of each product loaded into gel. Lane 1, 8 and 13 contains 1 µl, 0.5 µl and 0.25 µl 100 bp DNA ladder respectively.

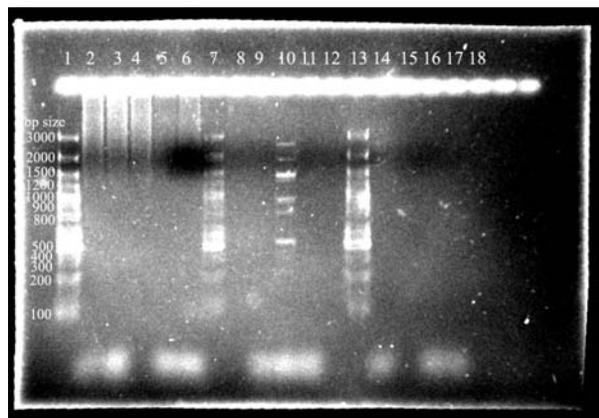


FIG. 8. Restriction Digest PCR for 3G. Lane 2-4 4ug/ µl BSA reaction mix; lane 5, 6 and 8 400 ng/ µl BSA reaction mix; lane 9, 10 and 12 0.2 µl DNA pol. buffer/rxn; lane 11,14,15 0.4 µl DNA polymerase buffer/rxn; lane 16-18 control PCR mix. Lane 2,5, 9,11,16 contains undigested human isolate; lane 3,6,10,14,17 contains Xba1 restriction endonuclease digested duck isolate. Lane 4, 8, 12, 15, 18 contains duck negative control. 15 µl of each product loaded into gel. Lane 1, 7 and 13 contains 1 µl 100 bp DNA ladder respectively.

DISCUSSION

As a starting point in optimization, literature conditions using colony PCR analysis were duplicated using *E. coli* isolates obtain from Trout Lake (goose, heron, gull, duck and human) and human clinical samples. Under literature conditions, faint but unique band patterns were obtained when 5µl of reaction product was loaded into the gel for visualization. To enhance the intensity of these bands, the PCR products from literature setting was further re-amplified for another 35 cycles. Although disappointing, the resulting

products produced intense smearing of bands but it was expected since this was an exponential increase of 35 PCR cycles on top of the initial 30 cycles. However, these bands provided optimism as they confirmed the possibility of using a nested PCR (re-amplification) technique with using the same primer to increase band intensity. Keeping in mind the purpose of BOX A1R PCR for DNA fingerprinting and the fact that this analysis would ultimately be used for high throughput screening, the goal of the optimization should be to improve band intensity and resolution in a single PCR reaction to reduce cost and time. The nature of using the first round PCR product for a second round of amplification was also taken into consideration because of its ability to carry forth non-primer specific products or artifacts for further amplification (17). The PCR was extended from 30 cycles in literature to 35. The extended number of cycles along with loading 15 µl reaction products into the gel and overnight exposure in EtBr did not result in any observable bands. This was unexpected since each of these conditions was designed to enhance band intensity. On the contrary, not only was there no improvement, the initial band patterns once observed were not present. Interpreting these results from the colony PCR, it was thought that perhaps variation in the amount of template yielded these discrepancies. In colony PCR, one complete isolated colony was sampled for PCR. As there were variations in colony size from colony to colony, starting DNA template would also vary. Since the ratio of primer to template is critical for the success of PCR reaction, especially in DNA fingerprinting, it could potentially account for the lack of results. This is because a ratio that is below optimum ratio promotes self-annealing (17) between the templates while a high ratio can encourage weak interactions resulting in non-specific PCR product such as primer dimers (15,17). *E. coli* isolates were cultivated and sampled from LB broth for further PCR analysis for the purpose of having better control over the PCR starting material. Samples taken from LB broth culture were of similar optical density and aliquoted in known volumes for greater stability in regards to reproducibility. However, initial cell suspension PCR reaction using 35 PCR cycles setting only produced one non-variant heavy band among all isolates tested. Further re-amplification using 100-fold diluted PCR products did not yield any bands. The dilution of the initial PCR product was done to prevent over-amplification and smearing of bands. The lack of results was thought to be due to either intrinsic factors of DNA templates that were limiting DNA amplification or inhibitory components of starting material preventing DNA amplification by Taq polymerase.

DMSO was previously shown in literature to possess the ability to destabilize nucleic acid (9,15). Because

of this properties, it was believed that the addition of DMSO would relieve torsional tension in areas of high G/C content (6), eliminate secondary structures (6) and decrease recombinant products (18). This might allow Taq polymerase access to areas that were once difficult to target due to factors such as high G/C content. DMSO has also been used to improve multiplex PCR reaction (15). Although multiplex PCR uses multiple primers while BOX PCR uses a single primer, both of these reactions target multi-loci positions. These findings support the use of DMSO for BOX AIR PCR. However, the PCR reactions with the addition of DMSO did not yield bands for any isolate and neither did the re-amplification of its products. This may be because DMSO lowers the melting and annealing temperature requirement during PCR reaction (14) and the PCR temperature steps used were programmed according to literature. Since these steps in literature were probably optimized without the use of DMSO, the inclusion of DMSO might potentially shift the reaction from the optimal conditions under these PCR temperature steps to a sub-optimal condition. Therefore, the notion of using DMSO for further analysis was dismissed because of the lack of results generated.

A common limitation with PCR analysis is the presence of inhibitory compounds (8,17). These inhibitors increase the susceptibility to errors, reduce assay resolution, and produce false results (12). PCR inhibitors must be diluted, inactivated or removed from sample to prevent it from affecting the PCR reaction (17). The samples in this study were prepared via heat lysis of *E. coli* cells to release the chromosomal DNA and polysaccharides on the bacteria may interfere with the release of DNA template from the cells (1). Proteinase might inhibit DNA polymerase enzyme itself (1) and mRNA might interact with the primer and decrease primer to DNA template ratio (20). Unknown components might interfere with the accessibility of the DNA template accessibility or binding of free Mg, which was demonstrated to be critical for successful PCR reaction (12). To decrease the possibility of inhibitory compounds from sample, the starting material was diluted ten-fold prior to PCR for cell suspensions and for colony PCR, half a colony (versus entire colony) was used. A lab strain isolate was also tested since lab strain *E. coli* tends to have thinner polysaccharide coats than wild type strains. Faint and unique band patterns were observed for each isolate. However, the bands were observed to be dumb-bell shaped and the resolution was low. A duplicate reaction using *E. coli* isolate of various hosts gave multiple unique band patterns except for goose isolate. This seemed to indicate that our initial problem with faint bands might have been due to interference from inhibitory compounds in the starting material.

A concern with the BOX PCR analysis was the

persistence dumb-bell shaped bands and low resolution. Clear, uniform and sharp bands are crucial if this analysis is to be used for MST. To investigate this problem, agarose gel preparation was carefully conducted where the heated agarose was observed under light to check for molten agarose (solid clumps) prior to casting the gel. Gel type was also investigated because it has been shown previously to limit DNA fingerprinting analysis (3). This is because the pore size can vary among same concentration but different types of agarose. Therefore, three types and brands of agarose available in-lab (Biorad, Amresco or Invitrogen) were tested. Unfortunately, the investigation did not result in improved band shape and resolution since all three types of agarose yielded similar results. Another possibility could be that the gel was not completely solidified in room temperature prior to usage. To test this theory, the agarose gels were left overnight in fridge and used the next day. The resolved banding patterns were cleaner and sharper from these gels when compared to previous gels.

To enhance resolution by producing uniform template, two restriction enzymes were used. Xba1 and Not1 both have larger target sequence (recognize 6 and 8 nucleotide sequence respectively) than other enzymes in-lab. Large target sequences were chosen because this suggests that there are less cleavage sites thus, a lower number of fragments and the fragments generated would be larger in size compared to fragments generated using other restriction enzymes. The rationale for restriction enzymes was to decrease the variation in the size of DNA products present in the agarose gels. By decreasing the variation in the DNA fragment size, fewer bands would be visualized after separation. Therefore, smears or fusions of bands with similar sizes would be minimized.

Initial PCR reactions using templates from restriction digests or undigested DNA subjected to gradual cooling did not yield bands. This might be because the ratio of DNA polymerase to primer/DNA complex is an important factor for PCR reactions. When this ratio is low, with the increase of amplicon via each cycle, the reaction would reach a point where it is no longer possible for one DNA polymerase to interact with each primer/ template (17). Variations in Taq polymerase concentration were also shown to influence PCR amplicons for DNA fingerprinting (20). Further reactions with twenty-fold dilution of original template and a two-fold increase in the concentration of original DNA polymerase was successful. Following this success, two other parameters were evaluated.

The initial lack of results may be due to change in sample preparation because the cells were concentrated two-fold from LB broth prior to heat lysis and the heat lysis step was also modified to gradually cool down. Because the starting material was concentrated two-

fold, DNA template might have increase. Previous studies have shown that the optimal primer: template ratio was sensitive to slight changes in the template or primer concentration (10,20). To ensure the use of the optimal primer: template ratio, these studies suggested titering DNA template against a constant primer concentration each time DNA extraction or quality was changed.

Aside from changing the primer: DNA template ratio, the increase in DNA concentration may also result in inefficient denaturation because the denaturation of DNA duplex can decrease when DNA concentration is high (10). Secondary structure formations were shown to influence DNA fingerprinting results (20). It was also previously proposed in literature that the BOX elements exist in secondary structures (2). Typical stem-loop structures could form between *boxA* and *boxC* for BOX elements as illustrated (FIG. 9).

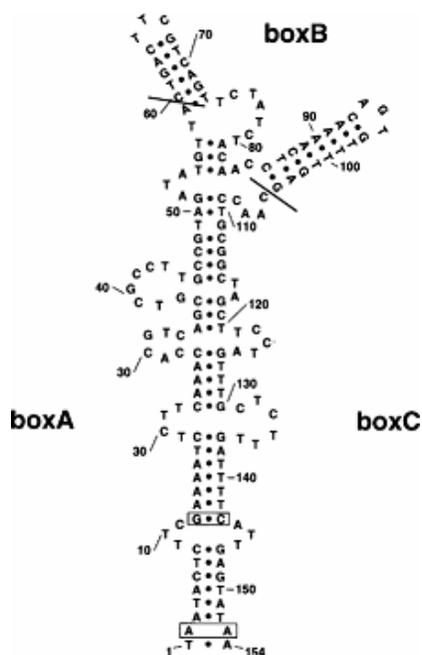


FIG. 9. BOX elements secondary structure (2).

Dilution series of starting material at twenty, forty and eighty-fold indicated that twenty and eighty-fold dilutions produced more bands from sample than forty-fold (2/5 positive sample versus 1/5). This is interesting, as it may suggest a decrease in amplification in the middle of the dilution series. However, all samples in the dilution series produced very faint bands. Perhaps the product concentrations from forty-fold dilution may be below the limit of detection in agarose gel. This is yet to be tested. To study this, a further duplication of the reaction that generates enough products for detection for at least 2/5

samples is required. Nonetheless, since the eighty-fold dilution worked as well as the twenty-fold dilution, it seemed to suggest that although dilution improved amplification, (twenty-fold versus ten-fold), improvement ceased after a certain optimal dilution.

Five different reaction conditions for the effect of storage buffer and BSA on the reaction were tested. Test conditions 4 $\mu\text{g}/\mu\text{l}$ BSA (1) and 400 $\text{ng}/\mu\text{l}$ BSA (13) were investigated because previous literature findings indicate that these conditions were able to enhance PCR by overcoming inhibition in complex samples such as feces, blood and meat. The additions of 0.2 μl and 0.4 μl DNA polymerase storage buffer to each reaction were also investigated since the original DNA polymerase was 0.2 μl per reaction. By increasing storage buffer volume in increments of 0.2 μl without increasing Taq polymerase, it would allow for the examination of the effects of the buffer itself. The fifth condition was a controlled reaction using previous conditions without additional additives.

Of the five conditions tested, 400 $\text{ng}/\mu\text{l}$ BSA seemed to be the most promising. It produced band patterns 2 out of 3 times and the band intensities under this condition were significantly stronger from the undigested DNA sample than the digested sample. It was speculated that the lid heating option set at 10°C above the highest temperature step (suggested by Biometra T-gradient PCR Thermocycler Operation Manual) might have caused reaction components to degrade during the PCR cycles. When the lid heating option was turned off, 400 $\text{ng}/\mu\text{l}$ BSA reaction failed to produce any band patterns in both 35 cycles and 40 cycles PCR analysis.

Fuzzy bands were also present at the bottom of each gel lane where samples were loaded. This might be due to the presence of primer dimers. Because primers are present at high concentrations, weak interactions can occur between them (4). Primer dimer formation can be reduced by careful primer design, the application of stringent conditions, the use of 'hot-start', and touch-down PCR (4) Cold start conditions have been shown in literature to give rise to every possible combination of primer dimers (4). Perhaps the PCR was programmed by the manufacturers to require heat lid option for normal hot start conditions, which would aid in removing non-template artifacts (17). By turning the heat lid option off 10 minutes into the PCR cycle, there was amplification that did not occur when the heat lid was turned off from the start (reaction conditions 3F and 3G). Further analysis of the BOX A1R primer for primer dimers using online primer analysis software (PrimerAnalyser) indicated a possibility of self-dimer formation in the 3' end under the maximum sensitivity setting. Figure 10 is a diagram of the possible self-dimer formation with BOX A1R primers.

Some differences from the literature include the use

of Taq polymerase from Invitrogen, increasing PCR cycle to 35 and 40, and the brand/type of thermocycler used. These differences could potentially influence the result of BOX A1R PCR as shown in literatures (20) with other amplification-based DNA fingerprinting analysis.

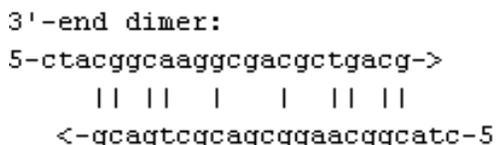


FIG. 10. 3'-end self-dimerization of BOX A1R primer from PrimerAnalyser.

Initial colony PCR analysis using literature conditions provided unique but faint band patterns with 30 cycles of PCR. Increasing colony PCR to 35 cycles did not yield any bands at first. However, when the starting material decreased (half a colony instead of entire colony), bands were observed. Thus, it was possible to obtain band patterns from both 30 and 35 cycles of PCR.

PCR reaction optimization with the addition of DMSO using literature temperature steps was not demonstrated in this experiment. Initial colony PCR (35cycles) and cell suspension PCR did not provide any bands. With half a colony taken, 10 fold dilutions for cell suspension PCR and twenty fold dilutions for restriction enzyme prepared samples, band patterns were observed. Further dilution of the restriction enzyme prepared samples (40 and 80 folds) did not improve band yields. Therefore, although dilutions were needed, further dilution might not be sufficient to improve band resolution.

Samples digested with restriction enzyme Xba1 or Not1 did not improve the band intensity or quality. Addition of Taq DNA polymerase storage buffer also did not improve product band yield. However, the reproducibility of PCR reaction improved (2 out of 3 PCR reaction versus 1 out of 3 PCR reaction for control condition) when 400 ng/μl BSA were included. The inclusion of BSA in PCR reaction might improve PCR reproducibility.

Various agarose type and brands were tested but did not improve band quality. Allowing gel to solidify at 4°C overnight prior to usage improved the band quality (shape and evenness). Therefore, the cooling of agarose gel before usage may increase the quality of band.

FUTURE EXPERIMENTS

Although a fully optimized set of conditions has not yet been established, parameters tested in this

experiment might give direction in reaching optimization. The most important step to take is to titer the DNA template against a constant primer concentration whenever there is a change in sample preparation. When dilution series with DNA template was performed, 40 fold dilutions dropped in performance compared to 20 fold and 80 fold dilutions. Additional experiments on dilution series with DNA template should be investigated to confirm if the decrease in performance is a reoccurring and significant event. BSA might aid in producing reproducible results while the use of restriction enzyme and DMSO might be discouraged or be given less priority during optimization. This study suggested the possibility of primer dimer formations. Future experimentation can investigate the effects of various primer concentration relative to DNA template via primer titrating.

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REFERENCES

1. Al-Soud, W.A., and P. Radstrom. 2000. Effects of amplification facilitators on diagnostic PCR in the presence of blood, feces, and meat. *J. Clin. Microbiol.* **38**: 4463-4470
2. Bernard, M., O. Humbert, M. Camara, E. Guenzi, J. Walker, T. Mitchell, P. Andrew, M. Prudhomme, G. Alloing, R. Hakenbeck, D. A. Morrison, G. J. Boulnois and J. Claverys. 1992. A highly conserved repeated DNA element located in the chromosome of *Streptococcus pneumoniae* *Nucleic Acids Res.* **20**:3479-3483
3. Berg, D. E., N. S. Akopyants, and D. Kersulyte. 1994. Fingerprinting microbial genomes using the RAPD or AP-PCR method. *Method Mol. Cell Biol.* **5**:13-24.
4. Brownie J., S. Shawcross, J. Theaker, D. Whitcombe, R. Ferrie, C. Newton, and S. Little. 1997. The elimination of primer-dimer accumulation in PCR. *Nucleic Acids Res.*, **25**: 3235-3241
5. Chen, Z. , and Y. Zhang. 2005. Dimethyl sulfoxide targets phage RNA polymerases to promote transcription. *Biochem. Biophys. Res. Comm.* **333**: 664-670
6. Choi, J., J. Kim, C. Joe, S. Kim, K. Ha and Y. Park. 1999. Improved cycle sequencing of GC-rich DNA template. *Exp. Mol. Med.* **31**: 20-24
7. Dombek, P. E., L. K. Johnson, S. T. Zimmerley, and M. J. Sadowsky. 2000. Use of repetitive DNA sequences and the PCR to differentiate *Escherichia coli* isolates from human and animal sources. *App. Environ. Microbiol.* **66**:2572-2577
8. Fredicks, D.N., and D.A. Relman. 1998. Improved amplification of microbial DNA from blood cultures by removal of the PCR inhibitor sodium polyanetholesulfonate. *J. Clin. Microbiol.* **36**: 2810-2816
9. Gouvea, V., R.I. Glass, P. Wood, K. Taniguchi, H.F. Clark, B. Forrester, and Z. Fang. 1990. Polymerase chain reaction amplification and typing of rotavirus nucleic acid from stool specimens. *J. Clin. Microbiol.* **28**: 276-282

10. **Hadrys, H., M. Balick, and B. Schierwater.** 1992. Applications of random amplified polymorphic DNA (RAPD) in molecular ecology. *Mol. Ecol.* **1**:55–63.
11. **Hassan, W. N., S. Y. Wang, and R. D. Ellender.** 2005. Methods to increase fidelity of repetitive extragenic palindromic PCR fingerprint-based bacterial source tracking efforts. *Appl. Environ. Microbiol.* **71**:512-518.
12. **Huggett, J.F., T. Novak, J.A. Garson, C. Green, S.D. Morris-Jones, R.F. Miller, and A. Zumla.** 2008. Differential susceptibility of PCR reactions to inhibitors: an important and unrecognized phenomenon. *BMC Res. Notes* **1**:70
13. **Kreader, C.A.** 1996. Relief of amplification inhibition in PCR with bovine serum albumin or T4 gene 32 protein. *Appl. Environ. Microbiol.* **62**: 1102–1106
14. **Markarian, S.A., A.M. Asatryan, K.R. Grigoryan, and H.R. Sargsyan.** 2006. Effect of diethylsulfoxide on the thermal denaturation of DNA. *Biopolymers.* **82**: 1–5
15. **Markoulatos, P., N. Sifakas, and M. Moncany.** 2002. Multiplex polymerase chain reaction: a practical approach. *J. Clin. Lab. Anal.* **16**:47–51
16. **1995. Microbial Source Tracking Guide Document.** *National Risk Management Research Laboratory Office of Research and Development U. S. Environmental Protection Agency Ohio: Cincinnati*
17. **Sachse, K., and J. Frey.** 2003. PCR Detection of Microbial Pathogens: Humana Press
18. **Shamma, F.V., R. Heikkila, and A. Oslanda.** 2001. Fluorescence-based method for measuring and determining the mechanisms of recombination in quantitative PCR. *Clin. Chim. Acta.* **304**: 19–28
19. **Stoeckel, D. M., and V. J. Harwood.** 2007. Performance, design, and analysis in microbial source tracking studies. *Appl. Environ. Microbiol.* **73**: 2405–2415.
20. **Tyler, K.D., G. Wang, S. D. Tyler, and W. M. Johnson.** Factors affecting reliability and reproducibility of amplification-based DNA fingerprinting of representative bacterial pathogens. *J. Clin. Microbiol.* **35**: 339-346