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Primer-Template Mismatch Does Not Contribute to Irreproducibility of PCR Replicates

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Primers incorporating a range of mutations and targeting an area of the lacZ gene were tested for variability among PCR replicates. The coefficient of variance obtained from 3 PCR replicates for each mutated primer was compared with that obtained using the non-mutated primer. A control amplicon was incorporated into each reaction; variability in the ratio of the control and test amplicon integrals was used as an indicator of replicate variance. Qualitative and quantitative analysis both indicated that primer-template mismatch does not contribute significantly to replicate variability. However, very few replicates were used in this study; a larger set should be tested to confirm these findings.

Microbial Source Tracking (MST) has applications in public health, the food and hospitality industries, water and environmental management. In order to protect public health for example, large water bodies frequented for recreation or sourced for public use are often tested for microbial contaminants, particularly fecal coliforms. Management of contaminants usually requires determination of their source, and for human waste specifically there are a whole array of potential methods to use (1, 2).

One such method, PCR 'fingerprinting', provides the potential for a rapid, simple and inexpensive identification of the likely source of a microbe. The BOX-A1R primer is often a primer of choice for PCR fingerprinting because of conservation of the template region and diversity in the amplicons produced. The BOX sequence was originally identified as a recurring inverted repeat in *Streptococcus pneumoniae*, containing 3 subparts, A, B and C. The BOX-A section was found to occur as a repetitive sequence in a wide range of Gram-positive and Gram-negative bacteria (3). Inversion and clustering of the BOX-A region allows a single primer, BOX-A1R, to generate range of product sizes in PCR with high diversity across and within species (4). Comparison of PCR products under particular reaction conditions with a library of fingerprints allows an organism and source to be identified (5).

However, various attempts at its use in distinguishing E. *coli* isolates between avian and human sources have met with limited success due to variability between replicates, enormous diversity within species and success bias caused by particular statistical techniques (6-8). Optimization studies have investigated factors ranging from template preparation to PCR temperature regimes (8-10). Lee and Wong (8) performed serial template dilution, restriction digests and inclusion of bovine serum albumin in the PCR mixture, Zhu (9) tested cell washing, alkaline lysis, heat lysis and DNA isolation while Yang and Yen (10) experimented with template growth phase and concentration, PCR annealing temperature and BSA once again. BSA was found to be beneficial at concentrations not greater than 400 ng/ul (8, 10). Cell washing, heat lysis and DNA isolation were all found to increase resolution of PCR bands (9), while annealing temperature adjustments were found to affect amplicon replication in a size-dependent manner (10). The other tests were not overtly beneficial.

Despite the work performed, rep-PCR using BOX-A1R is still not robust enough in the field. A major impediment to its use is its lack of reproducibility. The use of primer BOX-A1R to fingerprint a wide range of organisms increases the likelihood of a mutated target site. This is particularly true when the context of its principal use – Gram-negative *E. coli* – is compared to the organism in which it was developed – Gram-positive *S. pneumoniae*. Perhaps mismatch between primer and template contributes to variability between replicates. This was investigated by performing PCR using a unique target site on a template coupled with primers having varying degrees of mutation.

MATERIALS AND METHODS

Media, strains and growth conditions. *E. coli* K-12 strain MG1655 was used as the template culture for this project. It was grown overnight (15 – 21 h) at 37ºC on either LB agar or broth, using the same conditions and method as Zhu (9). 7.5 g of agar (Invitrogen, Cat. no. 30391-023) was added to produce the LB agar.

Primers selected. BLAST was performed for primers within the lacZ gene of *E. coli* K-12 strain MG1655 using NCBI PrimerBLAST (11). Two sets were chosen, having amplicon sizes of 356 bp and 669 bp, found on *E. coli* K-12 strain MG1655 genome at positions 364048-364697 and 365047-365382 respectively. The latter was used as a control amplicon (having primers C-r-JG12w and C-f-JG12w), from which the intensity of the test amplicon could be normalised. The former became the test amplicon; its reverse primer (T0-r-JG12w) remained constant, while its forward primer (T0-f-JG12w) was often substituted with primers having a range of mutations (T1.1-f-JG12w to T8-f-JG12w). Using the forward primer T0-f-JG12w as a base, a purpose-built application was used to create mutants according to a specified pattern and select those having the lowest primer-template melting temperature. Primers were then chosen manually from the filtered set. In general this pattern was a set number of mutations over either the 5' or 3' half of the primer, while nucleotides up to 3-5bpfrom the 3' were protected. Due to the melting temperature calculator relying on limited thermodynamic mismatch data (12-17), mutations were forced to be a minimum of 1 bp (but typically 2 or more bp) distant from

TABLE 1 Primers selected and their properties

Primer No.	IDT Ref. No.	Sequence (5'-->3')	No. Δ	Notes	Δposition
C-r-JG12w	89391284	cccgcaccgatcgcccttccc	0	Control amplicon: reverse primer	-
C-f-JG12w	89391283	accgacccagcgcccgttgc	0	Control amplicon: forward primer	-
T0-r-JG12w	89391285	accgcgcctttcggcggtga	0	Test amplicon: reverse primer	-
T0-f-JG12w	89391286	ggtgtcggctccgccgcctt	0	Test amplicon: Original forward primer	-
T1.1-f-JG12w	89391287	ggtgtcCgctccCccgcctt	2	Run of Cs	Whole primer
T1.2-f-JG12w	89391288	ggtgtcCgctccAccgcctt	2	A breaks run of Cs	''
T2.2-f-JG12w	89391289	ggCgtcCgctTcgcAgcctt	4	No Δs within 5bp from 3' end.	Whole primer
T3.1-f-JG12w	89391290	ggtgtcggctccAccAcctt	2	A breaks run of Cs	3' half
T3.2-f-JG12w	89391291	ggtgtcggctcAgcAgcctt	2	1 more bp clearance from 3' end over 3.1	''
T4-f-JG12w	89391292	ggtgtcggctTcAccAcTtt	4	Run of Cs broken.	3' half
T5.1-f-JG12w	89391293	ggtCtcCgctccgccgcctt	2	Large dangling end.	5' half
T5.2-f-JG12w	89391294	gCtgtcCgctccgccgcctt	2	Smaller dangling end.	''
T6.1-f-JG12w	89391295	gTtCtcCgcCccgccgcctt	4	Penetrance into middle of primer	5' half
T6.2-f-JG12w	89391296	gCtCtAgCctccgccgcctt	4	Less penetrance	''
T7.2-f-JG12w	89391297	ggtgtcCgctccgGcgcctt	2	Δs s.t. no run of more than 6 WC pairs occur. 6 bp from 3' end protected. (18)[1]	Whole primer
T8-f-JG12w	89391298	ggtgtcgCctccgccgcTtt	2	Δs s.t. 2 runs of more than 6 WC pairs occur. But Δ at bp 3 from 3' end. (19, 20)[1]	Whole primer

[1] (18) states that a run of 7 consecutive Watson-Crick base pairs hybridises much faster than a run of 6. However, (19) and (20) indicate that mismatches within 3 bp of the 3' end are much more significant than mismatches elsewhere, so the conflicting variables were juxtaposed to determine which was most significant. Qualitatively, they seemed equally insignificant, but more trials would be required to make a definitive judgment.

each other, as published data obtained (12-17) does not apply for consecutive mismatches. Primers were not initially tested for secondary structure formation and were obtained from IDT (individual reference numbers in Table 1).

Template preparation. Whole cell template preparation was adapted from Zhu's procedure (9): 750 ul of overnight *E. coli* culture (having an OD_{650} of 1.8) was centrifuged at 16000 x g for 1 min, the supernatant was discarded, cells were washed twice with 500 ul of 1 M NaCl and finally resuspended in 500 ul sterile water. Cells were extracted from half as much culture as Zhu (9) because the OD was approximately double. Also $1/10^{th}$ PCR inoculum was considered to give the best results (9), so washed cell suspension was made up to 500 ul rather than 50 ul.

PCR conditions. PCR was generally performed as follows: 1 ul of template, 2.5 ul of 10X PCR buffer (Invitrogen, P/N y02028), 1.5 ul of 50 mM $MgCl_2$ (Invitrogen, P/N y02016), 2 ul of 10 mM dNTPs, 1.25 ul at 20 uM for each primer used, 0.2 ul of 5U/ul Taq DNA Polymerase (Invitrogen, Cat. no. 18038-042, Lot no. 486001) and sterile water to a total volume of 25 ul. For hot start PCR, 10X Hot Start PCR buffer and Maxima Hot Start Taq DNA Polymerase (Fermentas, Ref. no. EP0602) were used instead of standard 10X PCR buffer and Taq DNA polymerase.10 mM dNTPs were prepared from a 100 mM dNTP set (Invitrogen, Cat. no. 10297-018). Lyophilised primers obtained from IDT were resuspended using sterile water to 100 uM, from which 20 uM working solutions were prepared. Master mixes were used wherever possible. 1/10 template volumes (0.1 ul template / 25 ul PCR mix) were often applied when using large volume master mixes to reduce the possibility for bacterial inactivation of the reaction. PCR was performed in either a Biometra T-Gradient PCR Thermocycler or Bio-Rad GeneCycler PCR Thermocycler. Initial cell lysis occurred at 94ºC, or 95ºC in the case of hot start PCR, for 9 min 30 s, followed by 35 cycles of denaturation, annealing and extension. Denaturation occurred at 94ºC, or 95ºC for hot start, for 30 s; annealing generally occurred at 60ºC for 30 s, though 45ºC and 50ºC were also used, as was touchdown PCR. Extension occurred at 72ºC for 1 min, with an extra 10 minutes at the final extension step.

Agarose gel electrophoresis. PCR products were separated on 1.5% agarose gel prepared using TopVision Agarose (Fermentas, Cat. no. R0491). 1X TAE buffer, used for both gel preparation and as running buffer, was diluted from a 10X laboratory stock. Sample and 6X loading dye were mixed in a 5:1 ratio, 8 ul of which was then loaded onto the gel. GeneRuler 100 bp Plus DNA Ladder (Fermentas, Ref. no. SM0321), GeneRuler 1kb DNA Ladder (Fermentas, Ref. no. SM0311) and Low Mass Ladder (Invitrogen, Cat. no. 10068-013) were used as standards with 0.5 - 1.0 ug usually loaded, though 1.4 ug (12 ul) was added in the case of Invitrogen Low Mass Ladder due to its skewed distribution (Figures 2 and 3). Electrophoresis was performed at approximately 7 V/cm after which gels were stained in 0.5 ug/ml ethidium bromide solution for 0.5 - 3 hours. Gels were then imaged under shortwave UV using Alpha Imager (Fisher Scientific).

Quantification using AlphaImager. In order to maximise sensitivity, images were exposed such that the most intense and relevant part of the image was close to saturation. PCR products of interest were manually selected using the rectangular boxing tool and intensity-area integrals were quantified using AlphaImager. Coefficient of variance (C.o.V.) was calculated for sets of replicates having the same primer and used to compare mutated primers with the original (T0-f-JG12w). Automatic backgrounding produced data which did not correlate well with qualitative judgments, and manually specifying the background using a second box was no more effective.

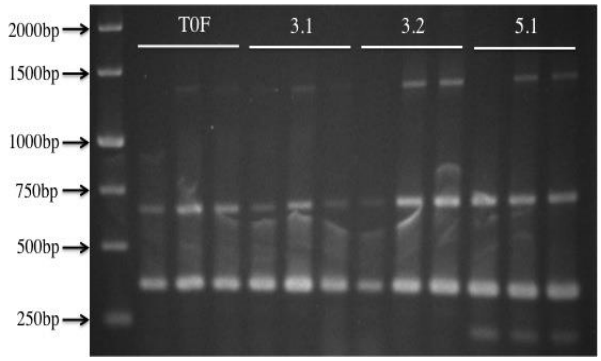

FIG 1 PCR having annealing temperature of 60ºC, using control primers C-r-JG12w, C-f-JG12w, reverse test primer T0-r-JG12w and forward test primers T0-f-JG12w, T3.1-f-JG12w, T3.2-f-JG12w and T5.1-f-JG12w, in triplicate. Fermentas GeneRuler 1 kb DNA Ladder is on the left.

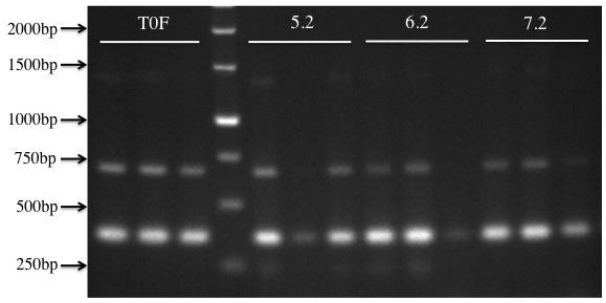

FIG 2 PCR having annealing temperature of 60ºC, using control primers C-r-JG12w, C-f-JG12w, reverse test primer T0-r-JG12w and forward test primers T0-f-JG12w, T5.2-f-JG12w, T6.2-f-JG12w and T7.2-f-JG12w, in triplicate. Fermentas GeneRuler 1 kb DNA Ladder is toward the left.

RESULTS

Effect of primer-template mismatch on PCR reproducibility. Qualitatively, Figure 1 shows primer T3.1-f-JG12w as more variable, T3.2-f-JG12w as equivalently variable and T5.1-f-JG12w as less variable than primer T0-f-JG12w, though artefacts on the gel might skew observations. Depending on whether the almost failed trials (2nd and 3rd trials of primers T5.2-f-JG12w and T6.2-f-JG12w respectively) in Figure 2 are retained or discarded, primers T5.2-f-JG12w and T6.2-f-JG12w might be much more variable or essentially the same as T0-f-JG12w. However, it is not really possible to judge variability from 2 samples. T7.2-f-JG12w seems slightly more variable than T0-f-JG12w in Figure 2. Other gels (data not shown) indicate that at an annealing temperature of 45ºC T1.1-f-JG12w is more variable than T0-f-JG12w, while T1.2-f-JG12w is less variable. Figure 3 meanwhile demonstrates a lesser variability on the part of primer T8-f-JG12w in comparison to T0-f-JG12w. Table 2 summarises quantitative results of primer variability.

PCR sensitivity. A template dilution assay was performed and it was found that the highest dilution for which a PCR product was obtained at 35 cycles was 1/100 of the standard template concentration (approximately 1/1000 of the template concentration used by Zhu (9)). A drop plate indicated that the density of the culture was 3 x 10^{11} cfu/ml, so a 1/100 dilution of the prepared template would correlate to about 5000 cfu. It seems surprising that the PCR would not occur below that template concentration (i.e. 500 cfu), but perhaps with more cycles a positive result would have been returned. In addition, perhaps DNA does not penetrate the cell wall effectively during heat lysis, resulting in low DNA template availability.

Effect of Hot Start PCR. Hot start PCR showed no advantages over the standard PCR procedure. Samples from standard and hot start PCR appeared equivalent.

DISCUSSION

Different melting curves with differing levels of certainty regarding the bound state of the primer-template pair – which partly describes the strength of the binding – suggest that at a temperature well below the T_M of the non-mutated primer, the mutated primer may be binding at e.g. 80±5% whereas the non-mutated primer would be binding at e.g. 98±1%. The difference in the uncertainties, particularly as a fraction of the binding, may be a principal cause of band inconsistency in BOX-A1R PCR fingerprinting.

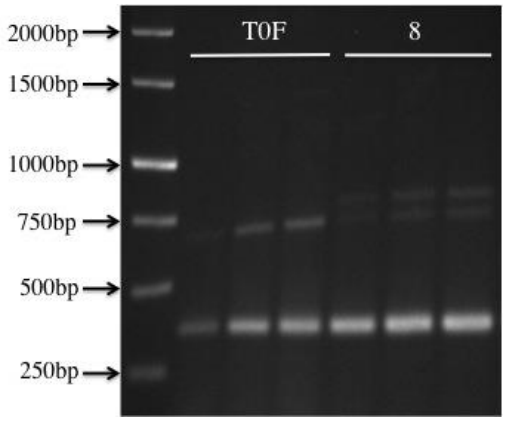

FIG 3 PCR having annealing temperature of 60ºC, using control primers C-r-JG12w, C-f-JG12w, reverse test primer T0-r-JG12w and forward test primers T0-f-JG12w and T8-JG12w, in triplicate. Fermentas GeneRuler 1 kb DNA Ladder is on the left. Gel irregularities are apparent on the left.

However, semi-quantitative analysis supported what was judged qualitatively –there was no difference in reproducibility between homologous and non-homologous primers. Table 2 shows that C.o.V. for most primers was *below* that of T0-f-JG12w, only T3.1-f-JG12w had a greater C.o.V. (50% more than T0-f-JG12w) and observing how the C.o.V.s themselves varied (e.g. C.o.V. of T5.1-f-JG12w was 50% *less* than T0-f-JG12w), it was well within the range of no significant difference. Nevertheless, the low number of replicates prevented statistical analysis from being particularly useful, especially when partially failed trials were discarded.

Comparison of the C.o.V.s for primer T0-f-JG12w across the 3 gels shows that clustering by gel occurs (Table 2). Using only a set of samples from a single gel results in a lower variation between samples than combining T0-f-JG12w samples from all gels. This suggests that the

TABLE 2 Summary of the variability of individual PCR reactions by primer, including background.

Gel	Primers	C.o.V	No. Δ
1	T0-f-JG12w	0.043	0
	T3.1-f-JG12w	0.064	2
	T3.2-f-JG12w	0.040	2
	T5.1-f-JG12w	0.020	2
2	T0-f-JG12w	0.056	0
	T5.2-f-JG12w	0.0227*	2
	T6.2-f-JG12w	0.0356*	4
	T7.2-f-JG12w	0.019	2
3	T0-f-JG12w	0.137	0
	T8-f-JG12w	0.020	2

*5.2.2 & 6.2.3 removed

procedure of electrophoresis and staining has a significant role to play in terms of variability between samples. Consideration of artefacts in Figures 3 and 5 lends further support to this idea.

A possible explanation is this system might be dominated by variability due to secondary structure in the reverse primer (T0-r-JG12w) at lower temperatures, e.g. if T0-r-JG12w was bound at 50±10% then it might be difficult to resolve differences between single-digit uncertainties in mutated and non-mutated primers. If this is not the case and the trials made are representative of a larger sample, then it would appear primer-template mismatch is not a significant source of variability in rep-PCR fingerprinting.

FUTURE DIRECTIONS

All of the gels had some sort of technical issue like smiling, bulging or surface drying. Some of the control PCRs were also inconsistent which impeded statistical work, and even interfered somewhat with a qualitative judgment. Surface drying problems could perhaps be remedied by pouring gels in a more humid environment although it was not a frequent problem. Partial failure of control PCRs perhaps could be remedied by performing a larger assay, allowing failed results to be discarded while still retaining enough to probe the significance of the hypothesis quantitatively.

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