Journal of Scientific Research & Reports
11(1): 1-9, 2016; Article no.JSRR.25029
ISSN: 2320-0227

SCIENCEDOMAIN *international*
www.sciencedomain.org

Optimization of Random Amplified Polymorphic DNA-PCR for Genotyping *Salmonella enterica* subspecies *enterica* serovar Typhi Using a Mathematical Approach

Ja'afar Nuhu Ja'afar[1,2*], Bhore Subhash Janardhan[3], Prabha Balaram[4] and Phua Kia Kien[2]

[1]Department of Biotechnology, Modibbo Adama University of Technology (MAUTECH), P.M.B. 2076, Yola, Adamawa State, Nigeria.
[2]Enteric Diseases Research Cluster, Institute for Research in Molecular Medicine (INFORMM), Universiti Sains Malaysia, 11800, USM, Penang, Malaysia.
[3]Department of Biotechnology, Faculty of Applied Sciences, AIMST University, 08100, Bedong, Kedah, Malaysia.
[4]Madathuvila Lane, Medical College, MRA 50, P.O, Trivandrum, 695011, Kerala, India.

Authors' contributions

This work was carried out in collaboration between all authors. Author JNJ designed the study, performed the experiment and wrote the first draft of the manuscript. Author BSJ managed the literature searches and analysed the results of the study. Author PB analysed the results of the study and wrote the second draft of the manuscript. Author PKK managed the experimental process and wrote the final draft of the manuscript. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JSRR/2016/25029
Editor(s):
(1) Yung-Fu Chang, Department of population Medicine and diagnostic Sciences, College of Veterinary Medicine, Cornell University, USA.
Reviewers:
(1) Charbell Miguel Haddad Kury, Medical School of the municipality of Campos dos Goytcazes, Brazil.
(2) Triveni Krishnan, National Institute of Cholera and Enteric Diseases, Kolkata, India.
Complete Peer review History: http://sciencedomain.org/review-history/14784

Original Research Article

Received 14th February 2016
Accepted 8th March 2016
Published 26th May 2016

ABSTRACT

Aim: A mathematical approach was employed to optimize and observe the interactive effects of Random Amplified Polymorphic DNA-PCR (RAPD-PCR) master mix with a view to resolving its limited reproducibility for reliable diagnostic and biomarker discovery for *Salmonella* Typhi.

**Corresponding author: E-mail: jnjaafar@yahoo.com;*

Methodology: A gradient PCR for RAPD primer annealing temperature was performed, and a rotatable centred central composite design (RCCCD) using Design Expert® software was used to generate 82 experiments with six replicated centre points. Master mix components optimized include concentrations of DNA template, PCR buffer, $MgCl_2$, RAPD primer, dNTPs and *Taq* DNA polymerase.
Results: The result of this study showed that significant interactions that yielded higher numbers of amplified DNA bands existed between PCR buffer and $MgCl_2$; dNTPs and $MgCl_2$; and RAPD primer and $MgCl_2$. Although not statistically significant, good interactive relationships that recorded higher numbers of bands were recorded between *Taq* with $MgCl_2$, and between RAPD primers and dNTPs. Reproducible RAPD-PCR results were obtained following the optimization with a co-efficient of variation (CV) value of 2.19%.
Conclusion: It was concluded that stringent and interactive master mix optimization is necessary if the simplicity and cost effectiveness of RAPD-PCR is to be utilized, and RSM offers a rapid and cost effective solution to this potentially tedious task.

Keywords: Salmonella Typhi; RAPD-PCR; genotyping; optimization; Kelantan; Malaysia.

1. INTRODUCTION

Typhoid fever is an acute systemic infection of humans of all ages caused by *Salmonella enterica* subspecies *enterica* serovar Typhi (*S.* Typhi). Only humans are carriers of the disease, causing about 21.6 million new cases and 200,000 deaths each year [1,2]. It is very difficult to get a true estimate of typhoid fever prevalence because only severe cases are reported and current diagnostic tests take 4-7 days [3]. An important strategy to curtail this persistent disease is to make available a fast and cost effective molecular tool for detection and epidemiological study of *S.* Typhi. One of the fundamental aspects in many molecular techniques is the amplification of specific DNA segments from the whole genome sequence using polymerase chain reaction (PCR). Random amplified polymorphic DNA-PCR (RAPD-PCR) is a type of DNA-based PCR method that is widely used as a DNA fingerprinting tool in various sectors of biomedical sciences including molecular epidemiology since the last two decades [4-6]. The method is simple, fast, cost-effective, and requires limited technical expertise. However, a major drawback of this method is its lack of reproducibility, from one laboratory to another, due to its inherent sensitivity to temperature and reagent variations; thus limiting its use in routine genotyping assays [7-10]. Therefore, resolving the lack of reproducibility of RAPD-PCR will ensure that this cost-effective technique will be a reliable diagnostic and biomarker discovery tool for molecular epidemiology of *S.* Typhi as well as for other diagnostic applications.

Even though the optimization of PCR is long practiced, there is, however, no single PCR protocol for all microbial species [11]. In RAPD-PCR, individual components have to be stringently optimized [12-17], suggesting the unsuitability of conventional PCR optimization method for RAPD-PCR. Previous studies have reported different optimization protocols by sequential variation of individual PCR parameters while keeping other parameters constant [7,18-23]. However, this method cannot resolve the lack of reproducibility nature of RAPD-PCR since multiple factors, including components of the PCR master mix, annealing temperatures, and PCR machine influence the outcome of the assay. In addition, there is limited literature describing the interactions between these master mix components. *Design of Experiment* is a systematic series of tests in which purposeful changes are made to input factors so that causes of significant changes in the output responses are clearly identified [24]. Response surface methodology (RSM) is a collection of mathematical techniques that are used for developing, improving, and optimizing experimental protocols [25].

The objective of this study was to address the problem of RAPD-PCR reproducibility by optimizing the assay components using central composite design (CCD) of the RSM to develop a tool for biomarker (DNA) discovery for *S.* Typhi.

2. MATERIALS AND METHODS

2.1 Bacterial Isolates

S. Typhi isolates, previously differentiated by pulsed-field gel electrophoresis (PFGE) and differing in district and year of isolation were

obtained from Hospital Universiti Sains Malaysia (HUSM), Kelantan, Malaysia.

2.2 RAPD Primers

All 20 decamer RAPD primers from Kit D, procured from Biospencer Malaysia, were used in this study. Seven primers have a melting temperature (Tm) of 32°C with 60% GC content, and the remaining 13 primers have a Tm of 34°C with 70% GC content.

2.3 Genomic DNA Extraction

This was carried out using QIAGEN® DNA extraction kit (DNeasy® Blood and Tissue Kit, USA), by following the manufacturer's guidelines. Briefly, one microlitre (µl) of overnight bacteria culture was pelleted and re-suspended in 180 µl ATL lysis buffer and 20 µl of proteinase K. Following incubation at 56°C for an hour in a water bath (Memmert, Germany), 200 µl AL lysis buffer was added, vortexed and incubated at 70°C for 10 min. The suspension was transferred into a QIAamp® spin column and centrifuged at 8,000 rpm for 1 min. The filtrate was discarded and 500 µl AW1 wash buffer was then added to the spin column and centrifuged at 8,000 rpm for 1 min. The filtrate was again discarded and a second wash and centrifugation step using AW2 wash buffer was done at 13,000 rpm for 5 min. Two hundred microlitres of AE elution buffer was added into the column to elute the bound DNA. Finally, the DNA concentration was measured spectrophotometrically using Nanodrop (NANODROP 2000c, USA).

2.4 Gradient PCR for RAPD Primer Annealing Temperature (Ta)

The RAPD-PCR method previously described was adopted [26]. The reaction was performed in a 25 µl total volume containing at a final concentration; 25 ng DNA template, 1X PCR buffer, 4 mM $MgCl_2$, 0.4 mM dNTP mix, 0.6 µM RAPD primer and 2.5 U *Taq* DNA polymerase (*Taq*). Distilled water was used to make up the final volume to 25 µl. The amplification was carried out in a programmable thermal cycler (MyCycler™ BIO-RAD, USA) with the following conditions: initial denaturation set at 94°C for 5 min; 45 cycles of 94°C for 30 sec, 38.5°C for 1 min and 74°C for 1 min; final extension at 74°C for 10 min. Fifteen microlitres of each PCR product was resolved in a 1.2% agarose gel and visualised in a UV fluorescence box (FluorChem®FC2, Alpha Innotech Corp, USA).

Following the screening, two primers, SBSD02 and SBSD05, representing other primers with Tm of 34°C and 32°C, respectively were used for the gradient PCR. For the initial round of gradient RAPD-PCR, a ±5°C from the individual primer's Tm was used [12]. Following agarose gel electrophoresis and resolution of the first PCR product, a new range of narrow temperatures was set based on the clarity and number of the PCR bands reproduced. A second gradient PCR was run and following agarose gel electrophoresis a new narrower range of temperatures was set. This cycle was repeated until the optimum Ta was obtained for both representative RAPD primers. The optimum Ta was used for optimizing the RAPD-PCR reactions.

2.5 Optimization of RAPD-PCR Master Mix

To determine the relationship and interaction between RAPD-PCR parameters systematically and experimentally, a mathematical approach using Design Expert® (Stat-Ease Inc., Minneapolis) was employed. A rotatable centred central composite design (RCCCD) of the RSM with five levels (-2, -1, 0, +1 and +2) was used to generate the experimental design. Independent variables optimized include DNA template, PCR buffer, $MgCl_2$, dNTPs, RAPD primers and *Taq* concentrations (Table 1). A set of 82 experiments with six replicated centre points was generated. All experiments were carried out in a final volume

Table 1. RAPD-PCR master mix concentrations and factorial levels

Parameters	Units	-2	-1	0	+1	+2
DNA template	ng	8.00	24.00	32.00	40.00	56.00
PCR buffer	X	0.40	1.00	1.20	1.50	2.00
$MgCl_2$	mM	0.30	1.20	1.70	2.20	3.10
dNTP	mM	0.08	0.26	0.36	0.46	0.64
RAPD primer	µM	0.20	0.39	0.50	0.60	0.80
Taq	U	0.50	1.00	1.25	1.50	2.00

of 25 µl each using distilled water to adjust the final volumes. The number of RAPD bands obtained for each experiment was considered as the response (γ). Prior to the use of the RAPD-PCR assay for further primer screening, the assay's reproducibility was performed as described previously for *S.* Enteritidis [27].

3. RESULTS

3.1 Optimization of Annealing Temperature (Ta)

To determine the optimum Ta for the RAPD primers, three rounds of gradient PCR using two representative RAPD primers, SBSD02 and SBSD05 with melting temperatures (Tm) of 34°C and 32°C, respectively, were performed. For the initial gradient RAPD-PCR, the Ta range was 30-39°C for primer SBSD02, and 30-37°C for primer SBSD05. Following resolution of the RAPD-PCR product in agarose gel, the second Ta range was narrowed to 35-37°C for primer SBSD02, and 34-36°C for primer SBSD05. A third Ta range that yielded the optimum was set at 36-37°C for primer SBSD02, and 34-35°C for primer SBSD05. Based on the maximum number of reproducible and sharp DNA bands obtained, the optimum Ta for primers SBSD02 and SBSD05 were determined to be 36.8°C and 34.8°C, respectively (Fig. 1). Hence, these Ta values were used in all subsequent RAPD-PCR experiments.

3.2 Optimization of RAPD-PCR Master Mix

To determine the optimum RAPD-PCR master mix, Design Expert® software was used to design the experiments. Independent factors: DNA template, PCR buffer, $MgCl_2$, dNTPs, RAPD primers and *Taq* concentrations, varied at five levels generated 82 experiments with six replicated centre points. The experimental results for all runs generated bands in the range of 2–23. The following second-order polynomial equation derived explains the relationship between dependent and independent variables.

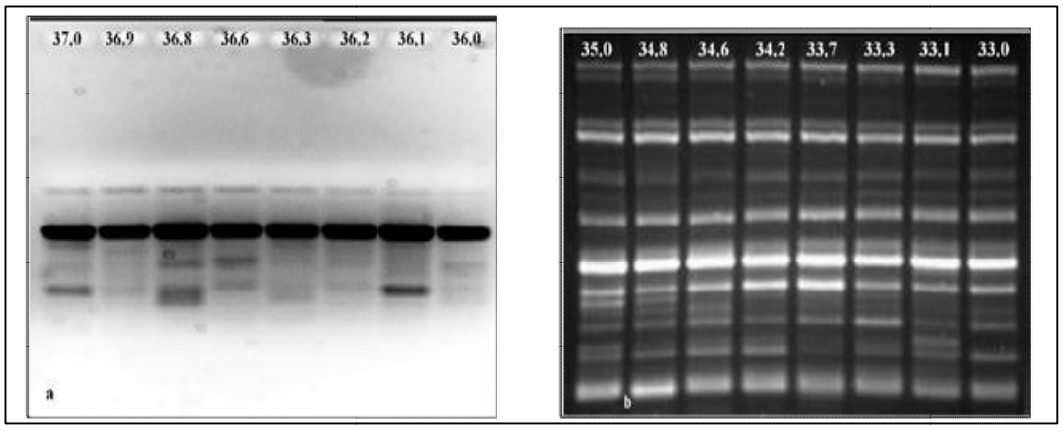

Fig. 1. Gel images showing the final gradient Ta results for primers SBSD02 and SBSD05. (a) Best Ta for primer SBSD02 was 36.8°C; gel image is shown in contrast to show the clarity of the DNA bands; (b) Best Ta for primer SBSD05 was 34.8°C

$$\left[\begin{array}{l}\gamma=\beta_o+\beta_1\chi_1-\beta_2\chi_2+\beta_3\chi_3-\beta_4\chi_4+\beta_5\chi_5+\beta_6\chi_6-\beta_{22}\chi_2^2-\beta_{33}\chi_3^2-\beta_{44}\chi_4^2-\\ \beta_{16}\chi_1\chi_6-\beta_{23}\chi_2\chi_3+\beta_{34}\chi_3\chi_4-\beta_{35}\chi_3\chi_5+\beta_{36}\chi_3\chi_6-\beta_{46}\chi_4\chi_6-\beta_{56}\chi_5\chi_6\end{array}\right] \quad (1)$$

where γ is the predicted dependent variable (number of bands); β_o is the intercept that measures the average response in the experiment; χ_1, χ_2, χ_3, χ_4, χ_5 and χ_6 are independent variables (DNA template, PCR buffer, $MgCl_2$, dNTPs, RAPD primers and *Taq* DNA polymerase concentrations, respectively). Furthermore, β_1, β_2, β_3, β_4, β_5 and β_6 are linear coefficients; β_{16}, β_{23}, β_{34}, β_{35}, β_{36}, β_{46} and β_{56} are interaction coefficients; and β_{22}, β_{33} and β_{44} are quadratic coefficients. In addition, the statistical model representing the predicted number of bands as a function of the independent

variables within the region under investigation is expressed by the following quadratic equation in coded form:

$$No.\ of\ Bands = 3.03 + 0.016A - 0.85B + 0.22C - 0.22D + 0.047E + 0.089F - 0.083B^2 - 0.13C^2 - 0.18D^2 - 0.12AF - 0.21BC + 0.26CD - 0.15CE + 0.12CF - 0.095DF - 0.10EF \quad (2)$$

where *A, B, C, D, E* and *F* are the coded variables for DNA template, PCR buffer, $MgCl_2$, dNTPs, RAPD primers and *Taq* DNA polymerase concentrations, respectively. Furthermore, the model evaluation was performed through analysis of variance (ANOVA) and coefficient of determination (R^2), which measures the goodness of fit of the regression model (Table 2). Similarly, the coefficient of variation (CV), which signifies the degree of precision with which the treatments were compared, was calculated.

The relationship between the response and the experimental levels of variables in the study were expressed in the form of three dimensional (3D) response surface plots (Fig. 2). Statistically significant interactions (*p<0.05*) were observed between PCR buffer and $MgCl_2$; dNTPs and $MgCl_2$; and RAPD primer and $MgCl_2$ concentrations. Fig. 2a showed that higher number of bands was obtained when 2.20 mM $MgCl_2$ and 1X PCR buffer were used. In addition, higher number of bands was obtained with 0.39 µM of RAPD primer and 2.20 mM of $MgCl_2$ (Fig. 2b). However, with dNTP, a low concentration of $MgCl_2$ at 1.70 mM was adequate for a significant interaction effect (Fig. 2c). Varying $MgCl_2$ concentration between 1.50 and 1.70 mM increased the number of bands, but a decreased effect ensued when the concentration exceeded 1.70 mM (Fig. 2c).

Although not statistically significant (*p>0.05*), good interaction effects were observed between $MgCl_2$ and *Taq*; dNTP and *Taq*; and RAPD primer and *Taq* (Fig. 2). Increased number of bands was observed with both high $MgCl_2$ and *Taq* concentrations (Fig. 2d). Low RAPD primer and high *Taq* concentrations were required to produce higher number of bands (Fig. 2e). Similarly, low dNTP and high *Taq* concentrations yielded higher number of DNA bands (Fig. 2f).

Based on the results, a ten-optimized numeric solution of different combinations of the master mix was generated. Fig. 3 shows the resolution of the RAPD-PCR products in 1.2% agarose gel when optimized concentrations of PCR master mix was used. For subsequent RAPD-PCR tests, a 25 µl reaction containing 40 ng DNA template, 1X PCR buffer, 2.2 mM $MgCl_2$, 0.4 mM dNTPs, 0.4 µM RAPD primer, and 1.25 U *Taq* DNA polymerase were used. Nuclease-free distilled water was used to adjust the final volume to 25 µl. Furthermore, a reproducibility test using the above master mix was performed by running three independent repeats of the RAPD-PCR assay using a single *S.* Typhi isolate (Fig. 3).

4. DISCUSSION

In an effort to improve the reproducibility of RAPD-PCR so that the advantages associated with it could be utilized for development of a tool for biomarker discovery in *S.* Typhi, we used RCCCD of the RSM to optimize the concentration of individual components of the master mix, and a gradient PCR to determine the optimum RAPD primer annealing temperature (Ta).

Table 2. ANOVA results for response surface quadratic model

	Sum of squares	DF*	Mean square	F value	**Prob> F
Quadratic model	82.25	16	5.14	15.29	<.0001
Lack of Fit	21.29	60	0.35	3.17	.0979
R^2 = 0.7901					
Adequate precision = 18.109					
Adjusted R^2 = 0.7384					
Predicted R^2 = 0.5764					
PRESS = 44.10					
CV = 2.19%					

**Degrees of freedom; **P<.05*

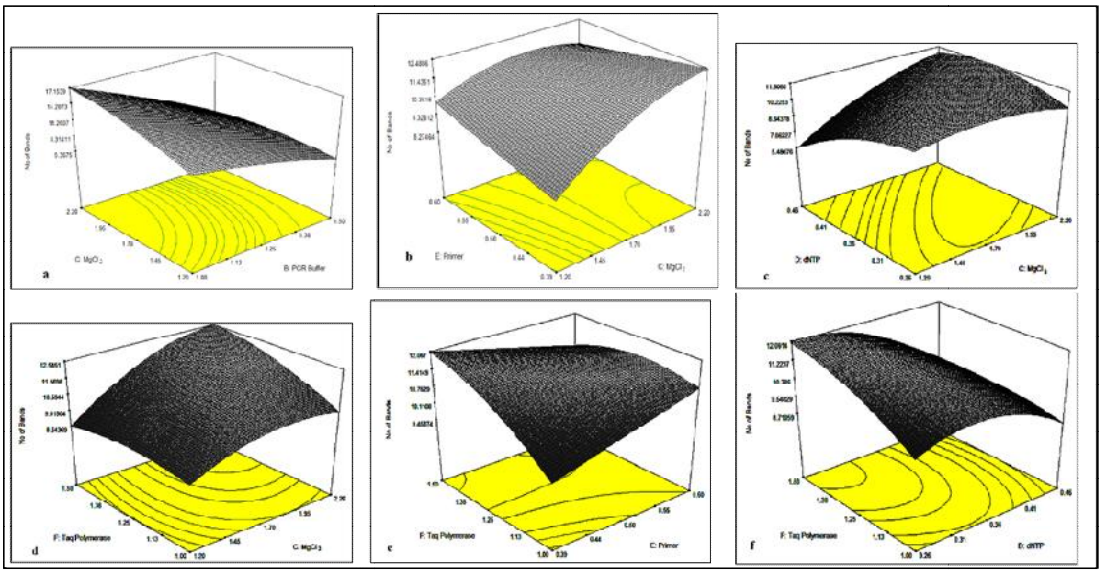

Fig. 2. Response surface plots showing the interactions of different master mix components in relation to the number of bands
(a) Effect of PCR buffer and $MgCl_2$ concentrations; (b) Effect of RAPD primer and $MgCl_2$ concentrations; (c) Effect of dNTP and $MgCl_2$ concentrations; (d) Effect of *Taq* and $MgCl_2$ concentrations; (e) Effect of *Taq* and RAPD primer concentrations; (f) Effect of *Taq* and dNTP concentrations

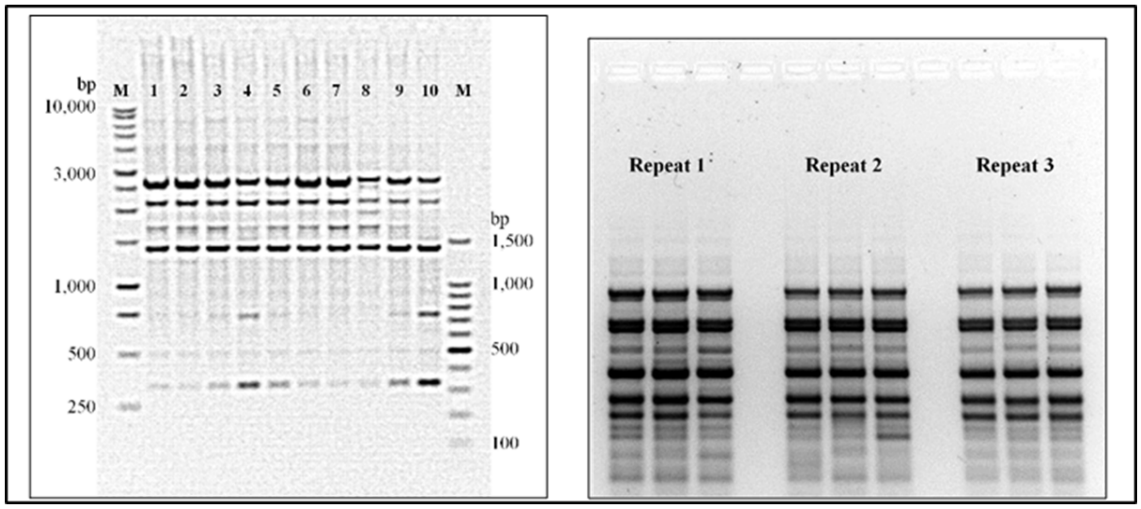

Fig. 3. Gel electrophoresis picture showing the results of the reproducibility test of the RAPD-PCR assay following optimization. (a) Result of RAPD-PCR assay using ten optimal conditions. (b)Three independent RAPD-PCR runs were carried out separately in triplicates

Due to the stringent nature of RAPD-PCR [28], this study started by optimizing the primer Ta of two primer categories *viz:* those having a melting temperature (Tm) of 32°C, represented by primer SBSD05, and those having a Tm of 34°C, represented by primer SBSD02. The optimum Ta obtained in this study were 36.8°C for primer SBSD02 and 34.8°C for primer SBSD05 (Fig. 1). It was observed that a 1°C change in Ta yielded a different band pattern, consistent with the sensitive nature of RAPD-PCR (Fig. 1a). Tingy et al. [29] have reported similar observations whereby a 1°C change in Ta affected DNA amplification significantly. More so, temperature

variations among thermal cyclers have been reported to affect PCR product amplifications [10,30,31]. Previous studies have reported varying RAPD primer Ta from as high as 42°C [32] to as low as 28°C [23]. Similarly, RAPD primer annealing times were varied from as long as 2 min [33] to as short as 30 sec [23]. In this study, 1 min annealing time was used for both RAPD primer categories. In contrast, the research findings of Innis and Gelfand [17] suggested that at a primer concentration of 0.2 µM, only a few seconds were needed for primer annealing. Similarly, Gelfand and White [34] showed optimum activity of *Taq* DNA polymerase even at very low primer Ta of 22°C.

A systematic analysis that explains the relationship between number of bands and master mix components, which was derived through mathematical modelling and analysis of variance (ANOVA) within the Design Expert® software, generated a second-order polynomial equation (expressed in both quadratic and coded forms) (Table 2 and Equations 1 and 2). Afterwards, model 3D graphs (Fig. 2) were plotted to examine the interactions and optima of the master mix. Results clearly suggest that higher number of bands can be obtained by using low buffer and high $MgCl_2$ concentrations (1X and 2.2 mM, respectively) (Fig. 2a). Increase in buffer or decrease in $MgCl_2$ concentrations led to less number of band amplification (Fig. 2a). Similarly, high concentrations of both $MgCl_2$ (2.2 mM) and RAPD primer (0.6 µM) (Fig. 2b) led to increased number of band amplification. These observations suggest that the concentration of $MgCl_2$ in the PCR reaction was at its optimum when the RAPD primer concentration was at 0.6 µM; increase or decrease of which can affect both DNA-DNA and DNA-protein interactions [15] leading to suboptimal DNA amplification. Devos and Gale [30] have demonstrated decreased primer annealing at higher magnesium ion concentrations. In contrast, Welsh and McClelland [4] showed that a higher level of magnesium ion was needed to stabilize primer and DNA-template interactions. It is pertinent to note that the requirement of magnesium ion, enzyme and primer concentrations in PCR amplifications vary from one microbial species to another [15, 17, 35]. Furthermore, sub-optimal magnesium ion concentration has been reported to affect primer annealing, PCR product specificity, primer-primer dimerization and *Taq* DNA polymerase activity [17]. Conversely, moderate $MgCl_2$ (1.7 mM) and dNTP (0.26 mM) concentrations used in this study recorded higher number of bands (Fig. 2c). This finding was in agreement with an earlier proposal by Innis and Gelfand [17] that in performing PCR, the total $MgCl_2$ concentration should be 0.5 to 2.5 mM greater than the total dNTP concentration. For example, a reaction should contain 0.9 to 2.9 mM $MgCl_2$ when 0.4 mM dNTP is used.

To illustrate further, even though not statistically significant, good interactions were observed between *Taq* and $MgCl_2$, and between dNTP and RAPD primer concentrations (Fig. 2). Using 1.5 U of *Taq* DNA polymerase, a higher number of bands were achieved with moderate $MgCl_2$ (1.7 mM), high RAPD primer (0.6 µM) and low dNTP (0.26 mM) concentrations, respectively. Ellsworth et al. [14] had recommended that to avoid artefact band patterns in RAPD-PCR fingerprinting, DNA template standardization is of critical importance. Similarly, Welsh and McClelland [13] had observed that a lack of reproducibility of RAPD-PCR fingerprinting was due to inadequate preparation of the DNA template. Despite the use of commercial kits for DNA extraction, varying DNA template concentrations in this study did not produce any significant result (not shown). Our observations were similar to the work of Shangkuan and Lin [36] who reported that DNA template concentrations over a wide range had no effect to RAPD-PCR band profiles.

5. CONCLUSION

In conclusion, the present study shows that improved reproducibility (CV =2.19%) of RAPD-PCR was observed following stringent master mix optimization, which was achievable through a mathematical approach. In addition, interactive optimization provides a clearer picture of the effects of individual PCR component on the number of bands produced rather than one factor at a time optimization, which has been the *status quo* in most laboratories. Additional genotyping studies on non-clinical isolates of *S.* Typhi using this optimized master mix might help to understand the exact significance of the mathematical approach in improving RAPD-PCR for discovery of biomarkers useful in diagnosis of *S.* Typhi infections, and perhaps permit the use of RAPD-PCR in the identification of new epidemiological markers for typhoid fever.

ACKNOWLEDGEMENTS

We wish to acknowledge the financial support provided by Universiti Sains Malaysia (USM) in

the form of Postgraduate Research Grant (PRGS) (Grant No. 1001/CIPPM/844078) and the Research University Cluster (RUC) project (Grant No. 1001/PSKBP/86300111).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Levine MM. Typhoid fever. In Bacterial infections of humans: Epidemiology and control. 4th ed.; Brachman PS, Abrutyn E, Eds. Springer New York, USA. 2009;913-938.
2. Crump JA, Luby SP, Mintz ED. The global burden of typhoid fever. Bulletin of the World Health Organization. 2004;82(5): 346-353.
3. Voetsch AC, Van Gilder TJ, Angulo FJ, Farley MM, Shallow S, Marcus R, et al. FoodNet estimate of the burden of illness caused by nontyphoidal *Salmonella* infections in the United States. Clinical Infectious Diseases. 2004;38(Supplement 3):S127-S134.
4. Welsh J, McClelland M. Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Research. 1990;18(24): 7213-7218.
5. Williams JG, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Research. 1990;18(22):6531-6535.
6. Caetano-Anolles G, Brant B, Gresshoff PM. DNA amplification fingerprinting using very short arbitrary oligonucleotide primers. Nature Biotechnology. 1991;9(6): 553-557.
7. Quintaes BR, Leal NC, Reis EMF, Hofer E. Optimization of randomly amplified polymorphic DNA-polymerase chain reaction for molecular typing of *Salmonella enterica* serovar Typhi. Revista da Sociedade Brasileira de Medicina Tropical. 2004;37(2):143-147.
8. Blixt Y, Knutsson R, Borch E, Rådström P. Inter laboratory random amplified polymorphic DNA typing of *Yersinia enterocolitica* and *Y. enterocolitica*-like bacteria. International Journal of Food Microbiology. 2003;83(1):15-26.
9. Dautle MP, Ulrich RL, Hughes TA. Typing and subtyping of 83 clinical isolates purified from surgically implanted silicone feeding tubes by random amplified polymorphic DNA amplification. Journal of Clinical Microbiology. 2002;40(2):414-421.
10. Penner G, Bush A, Wise R, Kim W, Domier L, Kasha K, et al. Reproducibility of random amplified polymorphic DNA (RAPD) analysis among laboratories. Genome Research. 1993;2(4):341-345.
11. Munthali M, Ford-Lloyd B, Newbury H. The random amplification of polymorphic DNA for fingerprinting plants. Genome Research. 1992;1(4):274-276.
12. Dassanayake RS, Samaranayake LP. Randomly amplified polymorphic DNA fingerprinting. In Pcr Protocols, 2nd ed.; JMS Bartlett; Stirling, D., Eds. Springer. 2003;226:117-122.
13. Welsh J, McClelland M. Fingerprinting using arbitrarily primed PCR: Application to genetic mapping, population biology, epidemiology and detection of differentially expressed RNAs. In The Polymerase Chain Reaction. Boston: Birklawer K, Mullis B, Ferre F, Gibbs RA, Eds. 1994;295-304.
14. Ellsworth D, Rittenhouse K, Honeycutt R. Artifactual variation in randomly amplified polymorphic DNA banding patterns. Biotechniques. 1993;14(2):214.
15. Blanchard M, Taillon-Miller P, Nowotny P, Nowotny V. PCR buffer optimization with uniform temperature regimen to facilitate automation. Genome Research. 1993;2(3): 234-240.
16. Linz U. Protocol optimization and the need for standardization of the polymerase chain reaction. Meth. Mol. Cell. Biol. 1991;2:98-102.
17. Innis MA, Gelfand DH. Optimization of PCRs. In PCR protocols: A guide to methods and applications. Innis MA, Gelfand DH, Sninsky JJ, White TJ, Eds. Academic Press Inc. 1990;3-12.
18. Weissensteiner T. Optimizing PCR with the aid of experimental design. In PCR technology: Current innovations, 2nd ed.; Weissensteiner T, Griffin HG, Griffin AM, Eds. CRC Press: USA. 2003;65-78.
19. Sahasrabudhe A, Deodhar M. Standardization of DNA extraction and optimization of RAPD-PCR. International Journal of Botany. 2010;6(3):293-298.
20. Zhang Y-P, Liu H-H. Optimization of RAPD-PCR system with orthogonal design in watermelon. Journal of Agricultural University of Hebei. 2005;4:012.

21. Jiao X, Wu F, Gao H. Optimization of RAPD conditions for soil microbes. Chinese Journal of Ecology. 2005;8:014.
22. Samal S, Rout G, Nayak S, Nanda R, Lenka P, Das P. Primer screening and optimization for RAPD analysis of cashew. Biologia Plantarum. 2003;46(2):301-304.
23. Hilton A, Banks J, Penn C. Optimization of RAPD for fingerprinting *Salmonella*. Letters in Applied Microbiology. 1997;24(4):243-248.
24. Anderson MJ, Whitcomb PJ. Response surface methods simplified: Optimizing processes using response surface methods for design of experiments. Productivity Press: New York, USA; 2005.
25. Myers RH, Montgomery DC, Anderson-Cook CM. Response surface methodology: Process and product optimization using designed experiments. 3rd ed.; John Wiley and Sons Inc.: New Jersey. 2009;1247.
26. Bhore SJ, Nurul A, Shah FH. Genetic variability based on randomly amplified polymorphic DNA in Kacip Fatimah (*Labisia pumila* Benth & Hook f) collected from Melaka and Negeri Sembilan States of Malaysia. Journal of Forest Science. 2009;25(2):93-100.
27. Betancor L, Schelotto F, Martinez A, Pereira M, Algorta G, Rodríguez MA, et al. Random amplified polymorphic DNA and phenotyping analysis of *Salmonella enterica* serovar Enteritidis isolates collected from humans and poultry in Uruguay from 1995 to 2002. Journal of Clinical Microbiology. 2004;42(3):1155-1162.
28. Erlich HA, Gelfand D, Sninsky JJ. Recent advances in the polymerase chain reaction. Science. 1991;252(5013):1643-1651.
29. Tingey SV, Rafalski JA, Williams JG. In Genetic analysis with RAPD markers, applications of RAPD technology to plant breeding: Joint plant breeding symposia series. Minneapolis, Minnesota, 1992;3-8.
30. Devos K, Gale MD. The use of random amplified polymorphic DNA markers in wheat. Theoretical and Applied Genetics. 1992;84:(5-6):567-572.
31. Klein-Lankhorst R, Vermunt A, Weide R, Liharska T, Zabel P. Isolation of molecular markers for tomato (*L. esculentum*) using random amplified polymorphic DNA (RAPD). Theoretical and Applied Genetics. 1991;83(1):108-114.
32. Kalkanci A, Saracli M, Guzel O, Yildiran S, Senol E, Kustimur S. Clustering of nosocomial *Candida kefyr* infections among hematological patients in a university hospital: Molecular typing of the strains by PFGE and RAPD. Journal de Mycologie Médicale/Journal of Medical Mycology. 2007;17(4):250-255.
33. Lim H, Lee KH, Hong C-H, Bahk G-J, Choi WS. Comparison of four molecular typing methods for the differentiation of *Salmonella* spp. International Journal of Food Microbiology. 2005;105(3):411-418.
34. Gelfand DH, White TJ. Thermostable DNA polymerases. In PCR protocols: A guide to methods and applications, Innis MA, Gelfand DH, Sninsky JJ, White TJ, Eds. Academic Press, Inc.: 1990;129-141.
35. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning. Cold spring harbor laboratory press New York: New York. 1989;2.
36. Shangkuan YH, Lin HC. Application of random amplified polymorphic DNA analysis to differentiate strains of Salmonella typhi and other *Salmonella* species. Journal of Applied Microbiology. 1998;85(4):693-702.

Peer-review history:
The peer review history for this paper can be accessed here:
http://sciencedomain.org/review-history/14784