...KAAN HAMDAN TAHIR
...VERSITI SAINS MALAYSIA

RUJUKAN

UNIVERSITI SAINS MALAYSIA
GERAN PENYELIDIKAN UNIVERSITI PENYELIDIKAN
LAPORAN AKHIR

DEVELOPMENT OF A NOVEL APPROACH MULTIPLEX LATE-PCR ELECTROCHEMICAL-ENZYME BASED DNA SENSOR FOR SEQUENCE SPECIFIC DETECTION OF VIBRIO CHOLERAE

PENYELIDIK

DR. CHAN YEAN YEAN

2015

End of Project Report For ScienceFund

A. Description of the Project

1. **Project number** : 02-01-05-SF0471
2. **Project Title** : Development of a Novel Approach Multiplex LATE-PCR Electrochemical-Enzyme Based DNA Sensor for Sequence-Specific Detection of Vibrio cholerae
3. **Project Leader** : Chan Yean Yean
4. **Project Team** :

   (please provide an assessment of how the project team performed and highlight any significant departures from plan in either structure or actual man-days utilised)

   NIL
5. **Industrial Partnership** :

   (Please describe the nature of collaborators with relevant industry)

   NIL
6. **National/International Collaboration** :

   (Please identify research organisations and describe the nature of collaboration)

   NIL
7. **Project Duration** : 24(months)

   Start date : August (month) 2011 (year)

   End date : July (month) 2013 (year)
8. **Total Budget Approved** : RM 252500

Tutup Geran.

PROF. MADYA LEE KEAT TEONG
Pengarah
Pejabat Pengurusan & Kreativiti Penyelidikan
Universiti Sains Malaysia
11800 USM, Pulau Pinang.

B. Objectives of the Project

1. Socio-economic Objectives (SEO)

Which socio-economic objectives are addressed by the project? (Please identify the Research Priority Area, SEO Category and SEO Group under which the project falls. Refer to the Malaysian R&D Classification System, 4th Edition.

Research Priority Area : BIOTECHNOLOGY Sub Cluster :

SEO Category : Natural Sciences, Technologies and Engineering

SEO Group : Biotechnology

SEO Area : Biotechnology

2. Fields of Research (FOR)

Which are the two main FOR Categories, FOR Groups, and FOR Areas of your project? (please refer to the Malaysian R&D Classification System, 4th Edition)

a. Primary field of research

FOR Category : Biotechnology

FOR Group : Biotechnology

FOR Area : Diagnostics

b. Secondary Field of research

FOR Category : Engineering Sciences

FOR Group : Interdisplinary Engineering

FOR Area : Nanotechnology

C. Objective Achievement

- **Original project objectives**

(Please state the specific project objectives as described in Section II of the Application Form)

Phase 1: Development of a Multiplex Linear-After-The-Exponential (LATE)- Polymerase Chain Reaction (PCR)
- To design specific primers and optimize a multiplex LATE-PCR which is capable of simultaneously detecting the presence of V. cholerae, as well as cholera toxin and internal control genes.

Phase 2: Development of a Mixed Self-Assembled Monolayer (SAM) on Disposable Screen-Printed Gold Electrode (SPGE)
- To design thiol-modified capture probe for sequence-specific hybridization with target LATE-PCR amplicons.
- To optimize the immobilization of capture probe DNAs on gold electrode surface using Self-Assembled Monolayer (SAM) system.

Phase 3: Development of a Multiplex Electrochemical-Enzyme based DNA sensor
- To optimize the hybridization of labeled LATE-PCR amplicons to the capture probe DNAs.
- To optimize the simultaneous detection of multiple redox reactions catalyzed by alkaline phosphatase and horseradish peroxidase by amperometry.

Phase 4: Evaluation of the Multiplex Electrochemical-Enzyme based DNA sensor
- To perform analytical evaluation of the multiplex electrochemical DNA hybridization genosensor.
- To evaluate the performance of the multiplex electrochemical DNA hybridization genosensor using clinical samples.

- **Objective Achieved**

(Please state the extent to which the project objectives were achieved)

Phase 1: Development of a Multiplex Linear-After-The-Exponential (LATE)- Polymerase Chain Reaction (PCR)
- To design specific primers and optimize a multiplex LATE-PCR which is capable of simultaneously detecting the presence of V. cholerae, as well as cholera toxin and internal control genes - 100% achieved as per planned

Phase 2: Development of a Mixed Self-Assembled Monolayer (SAM) on Disposable Screen-Printed Gold Electrode (SPGE)
- To design thiol-modified capture probe for sequence-specific hybridization with target LATE-PCR amplicons.- 100% achieved as per planned
- To optimize the immobilization of capture probe DNAs on gold electrode surface using Self-Assembled Monolayer (SAM) system - 100% achieved as per planned

Phase 3: Development of a Multiplex Electrochemical-Enzyme based DNA sensor
- To optimize the hybridization of labeled LATE-PCR amplicons to the capture probe DNAs - 100% achieved as per planned
- To optimize the simultaneous detection of multiple redox reactions catalyzed by alkaline phosphatase and horseradish peroxidase by amperometry - 100% achieved as per planned

Phase 4: Evaluation of the Multiplex Electrochemical-Enzyme based DNA sensor
- To perform analytical evaluation of the multiplex electrochemical DNA hybridization genosensor - 100% achieved as per planned
- To evaluate the performance of the multiplex electrochemical DNA hybridization genosensor using clinical samples - 100% achieved as per planned

- **Objectives not achieved**

(Please identify the objectives that were not achieved and give reasons)

NIL

D. Technology Transfer / Commercialisation Approach, if any.

(Please describe the approach planned to transfer/commercialise the results of the project)

This study has successfully developed a novel strategy for detecting two target DNA amplicons simultaneously on a screen-printed electrode with dual gold working electrodes. The LATE-PCR primers and probes immobilized on the electrodes can be customized for the detection of other pathogenic organisms and virulence genes.

The use of gold electrode substantially simplifies the method that is used for functionalizing the electrode and this will promote the use of biosensor in laboratory for detection of PCR amplicons.

The outcome of this project include high-impact publications, book chapter, international and national conference presentations and patent filed at the intellectual property corporation of Malaysia (myIpo). Date of filling: 16 Feb 2012

E. Assessment of Research Approach

(Please highlight the main steps actually performed and indicate any major departure from the planned approach or any major difficulty encountered)

NIL

F. Assessment of the Project Schedule

(Please make any relevant comment regarding the actual duration of the project and highlight any significant variation from plan)

NIL

G. Assessment of Project Cost

(Please comment on the appropriateness of the original budget and highlight any major departure from the planned budget)

NIL

H. Additional Project Funding Obtained

(In case of involvement of other funding sources, please indicate the source and total funding provided)

NIL

I. Benefits of the Project

(please identify the actual benefits arising from the project as defined in Section III of the Application form. For examples of outputs, organisational outcomes and sectoral/national impacts, please refer to Section III of the Guidelines for the Application of R&D Funding under ScienceFund)

1. Direct Outputs of the Project

(Please describe as specifically as possible the outputs achieved and provide an assessment of their significant to users)

i. Technical contribution of the project

a. What was the achieved direct output of the project :

For basic oriented research projects?

- [ ] Algorithm
- [ ] Structure
- [X] data
- [ ] Other, please specify :

For applied research (technology development) projects :

- [X] Method/technique
- [X] Demonstrator/prototype
- [ ] Product/component
- [ ] Process
- [ ] Software
- [ ] Other, please specify:

b. How would you characterise the quality of this output?

- [ ] Signifcant breakthrough
- [X] Major improvement
- [ ] Minor Improvement

ii Contribution of the project to knowledge

a. How has the output of the project been documented

- [X] Detail project report
- [X] Products/process specification documents
- [ ] Other, please specify :

b. Did the project create an intellectual property stock?

- [ ] Patent obtained
- [X] Patent pending
- [ ] Patent application will be filed
- [ ] Copyright

c. What publications are available?

			National	International
X	Article(s) in scientific publications	How many :	0	2
X	Paper(s) delivered at conferences/seminars	How many :	3	3
X	Book	How many :	0	1
	Other, please specify :			

d. **How significant are citations of the results?**

[ ]	Citations in national publications	How many : 0
[X]	Citations in international publications	How many : 2
[ ]	Not yet	
[ ]	Not known	

2. **Organisational Outcomes of the Project**

(Please describe as specifically as possible the organisational benefits arising from the project and provide an assessment of their significance)

i. **Contribution of the project to expertise development**

a. **How did the project contribute to experties?**

[X]	PhD degrees	How many : 2
[ ]	MSc degrees	How many : 0
[X]	Research staff with new specialty	How many : 1
[ ]	Other, please specify :	

b. **How Significant is this expertise?**

- [X] One of the key areas of priority for Malaysia
- [ ] An important area, but not a priority one

ii. **Economic contribution of the project?**

a. **How has the economic contribution of the poroject materialised?**

- [ ] Sales of manufactured product/equipment
- [ ] Royalties from licensing
- [ ] Cost savings
- [X] Time savings
- [ ] Other, please specify :

b. **How important is this economic contribution?**

[ ]	High economic contribution	How many : RM0
[X]	Medium economic contribution	How many : RM0
[ ]	Low economic contribution	How many : RM0

c. **When has this economic contribution materialised?**

- [ ] Already materialised
- [ ] Within months of project completion
- [ ] Within three years of project completion
- [X] Expected in three years or more
- [ ] Unknown

iii. Infrastructural contribution of the project

a. What infrastructure contribution has the project had?

- [ ] New equipment — Value : RM0
- [X] New/improved facility — Investment : RM12800.00
- [ ] New information networks
- [ ] Other, please specify :

b. How significant is this infrastructure contribution for the organisation?

- [ ] Not significant/does not leverage other projects
- [X] Moderately significant
- [ ] Very significant/significantly leverages other projects

iv. Contribution of the project to the organisation's reputation

a. How has the project contributed to increasing the reputation of the organisation

- [ ] Regconition as a Center of Excellence
- [ ] National award
- [ ] International award
- [X] Demand for advisory services
- [X] invitations to give speeches on conferences
- [X] Visits from other organisations
- [ ] Other, please specify :

b. How important is the project's contribution to the organisation's reputation?

- [ ] Not significant
- [ ] Moderately significant
- [X] Very significant

3. National Impacts of the project

(If known at this point in time, please describe as specifically as possible the potential sectoral/national benefits arising from the project and provide an assessment of their significance)

i. Contribution of the project to organisational linkages

a. Which kinds of linkages did the project create?

- [X] Domestic industry linkages
- [ ] International industry linkages
- [X] Linkages with domestic research institutions, universities
- [X] Linkages with international research institutions, universities

b. What is the nature of the linkages?

- [ ] Staff exchanges
- [X] inter-organisational project team
- [X] Research contract with a commercial client
- [X] Informal consultation
- [ ] Other, please specify :

ii. **Social-economic contribution of the project**

a. **Who are the direct customer/beneficiaries of the project output?**

Customers/beneficiaries	Number
Total	

b. **How has/will the socio-economic contribution of the project materialised?**

- [X] Improvements in health
- [ ] Improvements in safety
- [X] improvements in the environment
- [ ] Improvements in energy consumption/supply
- [X] Improvements in international relations
- [ ] Other, please specify :

c. **How important is this socio-economic contribution?**

- [ ] High social contribution
- [X] Medium social contribution
- [ ] Low social contribution

d. **When has/will this social contribution materialised?**

- [ ] Already materialised
- [ ] Within three years of project completion
- [X] Expected in three years or more
- [ ] Unknown

Date :	**Signature :**

END OF PROJECT & BENEFITS REPORT

ase fill in the relevant details in the space provided.
ase tick (/) where appropriate and provide further description, if required.

0 PROJECT DESCRIPTION

.	Project Number	02-01-05-SF0471	
.	Project Title	Development of a Novel Approach Multiplex LATE-PCR Electrochemical-Enzyme Based DNA Sensor for Sequence-Specific Detection of *Vibrio cholerae*	
.	Project Leader's Details	Name: Chan Yean Yean	
		Position: Senior Lecturer	Address: Department of Medical Microbiology and Parasitology, School of Medical Sciences, Health Campus, Universiti Sains Malaysia
		Email: yeancyn@yahoo.com Contact No: (off); 09-767 6258 (h/p) 012-901 1066	
.	Project Funding	Total Budget Approved: RM 252,500.00 Total Project Expenses : RM 247,609.53	
.	Project Time Frame (According to agreement)	Start Date : : 8 months / 2011 year ; Completion Date : : 7 months / 2013 year Duration : 24 months	
.	Project Extension (approval from MOSTI; if applicable;)	1st Extension : 6 months; Revised completion date : 1 months / 2014 year 2nd Extension : months; Revised completion date : ___months / ___year	

2.0 OBJECTIVES ACHIEVEMENT

Problem Statement (A brief and precise description)		Currently, the detection and identification of *V. cholerae* rely heavily on conventional culturing technique which is laborious, time consuming and require 2-3 days to get resu Therefore, electrochemical DNA biosensor is one of the strategies being explored for t development of DNA biosensors as portable handheld devices as the electrochemical technology is amenable to miniaturisation, can be accurate and sensitive with simple s contained instrumentation and easily controlled reaction conditions
a.	**Original Project Objective** (Based on MOSTI approval)	Phase 1: Development of a Multiplex Linear-After-The-Exponential (LATE Polymerase Chain Reaction (PCR) • To design specific primers and optimize a multiplex LATE-PCR whi capable of simultaneously detecting the presence of V. cholerae, as as cholera toxin and internal control genes. Phase 2: Development of a Mixed Self-Assembled Monolayer (SAM) on Disposable Screen-Printed Gold Electrode (SPGE) • To design thiol-modified capture probe for sequence-specific hybridization with target LATE-PCR amplicons. • To optimize the immobilization of capture probe DNAs on gold elec surface using Self-Assembled Monolayer (SAM) system. Phase 3: Development of a Multiplex Electrochemical-Enzyme based DN sensor • To optimize the hybridization of labeled LATE-PCR amplicons to the capture probe DNAs. • To optimize the simultaneous detection of multiple redox reactions catalyzed by alkaline phosphatase and horseradish peroxidase by amperometry. Phase 4: Evaluation of the Multiplex Electrochemical-Enzyme based DN sensor • To perform analytical evaluation of the multiplex electrochemical DI hybridization genosensor. • To evaluate the performance of the multiplex electrochemical DNA hybridization genosensor using clinical samples.
b.	**Original Achieved** (Please state the extent to which the project objectives were achieves)	Phase 1: Development of a Multiplex Linear-After-The-Exponential (LAT Polymerase Chain Reaction (PCR) • To design specific primers and optimize a multiplex LATE-PCR wh capable of simultaneously detecting the presence of V. cholerae, a as cholera toxin and internal control genes – **100% achieved as pe planned** Phase 2: Development of a Mixed Self-Assembled Monolayer (SAM) on Disposable Screen-Printed Gold Electrode (SPGE) • To design thiol-modified capture probe for sequence-specific hybridization with target LATE-PCR amplicons – **100% achieved a planned** • To optimize the immobilization of capture probe DNAs on gold elec surface using Self-Assembled Monolayer (SAM) system – **100% achieved as per planned** Phase 3: Development of a Multiplex Electrochemical-Enzyme based DI sensor • To optimize the hybridization of labeled LATE-PCR amplicons to th capture probe DNAs – **100% achieved as per planned** • To optimize the simultaneous detection of multiple redox reactions catalyzed by alkaline phosphatase and horseradish peroxidase by

	amperometry – **100% achieved as per planned** Phase 4: Evaluation of the Multiplex Electrochemical-Enzyme based DNA sensor • To perform analytical evaluation of the multiplex electrochemical DNA hybridization genosensor – **100% achieved as per planned** • To evaluate the performance of the multiplex electrochemical DNA hybridization genosensor using clinical samples – **100% achieved as per planned**
Original Not Achieved (Please state the objectives that were no achieved and provide reason)	NIL

.0 ASSESSMENT OF RESEARCH APPROACH

Please highlight the main steps actually performed and indicate any major departure from the planned approach or any major difficult ncountered)

IL

.0 ASSESSMENT OF PROJECT SCHEDULE

Please make a relevant comment regarding the actual duration of the project and highlight any significant variation from plan)

IL

0 ASSESSMENT OF PROJECT COSTS

Please comment on the appropriateness of the original budget and highlight any major departure from the planned budgets)

IL

B) BENEFITS OF THE PROJECT

1.0 What was the achieved direct output of your project?

[√] Method/technique /process (Please describe)

The method to functionalize screen-printed gold electrode bisensor (SPGEB) with mixed self-assembled monolayer of DNA capture probes and MCH has been developed. Applicability of the developed DNA sense and the method of detection have been shown using synthetic targets, puri genomic DNA, bacterial cells and spiked stool samples.

[√] Demonstrator/laboratory prototype (Please describe)

The DNA sensor has been developed as a laboratory prototype.

[ ] Product/component (Please describe)

[ ] Software (Please describe)

[ ] Others, Please specify:

2.0 What intellectual property (IP) did your project acquire?

		International	National
[ ]	Patent granted Please state Invention Title & Number: Inventor:	[ ] quantity	[ ] quantity
[√]	Patent pending Please state Invention Title & Number: Device and method for detection of nucleic acid(s) PI 2012000667 Inventor: Chan, Yean Yean, Ang, Geik Yong & Yu, Choo Yee	[ ] quantity	[1] quantity
[ ]	Potential patent to be filed Please state Invention Title & Number: Inventor:	[ ] quantity	[ ] quantity
[ ]	Copyright Please state Invention Title & Number: Inventor:	[ ] quantity	[ ] quantity
[ ]	Trade Mark Please state Invention Title & Number: Inventor:	[ ] quantity	[ ] quantity
[ ]	Industrial Design Please state Invention Title & Number: Inventor:	[ ] quantity	[ ] quantity
[ ]	Others, please specify: ______		

3.0 What publications did your project acquire?

		International		National	
√	Article(s) in scientific publications	2	quantity		quantity

Please state

Title: Multiplex electrochemical genosensor for identifying toxigenic *Vibrio cholerae* serogr O1 and O139
Publication: Chemical Communications (Cambridge)
Author(s): Yu, Choo Yee, Ang, Geik Yong & Chan, Yean Yean
Year: 2013

Title: Ambient temperature detection of PCR amplicons with a novel sequence-specific nu acid lateral flow biosensor
Publication: Biosensors and Bioelectronics
Author(s): Ang, Geik Yong, Yu, Choo Yee & Chan, Yean Yean
Year: 2012

- ***Kindly please attached the articles (softcopy & hardcopy) ; The contribution of MOSTI as the fund pr must be acknowledged in the publications.***

		International		National	
√	Paper(s) delivered at conference/seminar	3	quantity	3	quantity

Please state

Title: An integrated electrochemical genosensing platform based on dry reagent multiplex PCR strategy.
Conference: AsiaSense: The 6th International Conference on Sensors
Author(s): Yu, Choo Yee, Ang, Geik Yong & Chan, Yean Yean
Year: 2013

Title: Development of a multiplex LATE-PCR-coupled electrochemical genosensor platfor cholera.
Conference: National Biotechnology Seminar
Author(s): Yu, Choo Yee, Ang, Geik Yong & Chan, Yean Yean
Year: 2013

Title: A multiplex electrochemical genosensor for cholera using disposable screen-printed electrode bisensors.
Conference: 3rd International Conference on Bio-sensing Technology
Author(s): Yu, Choo Yee, Ang, Geik Yong & Chan, Yean Yean
Year: 2013

Title: A dual-acting PCR stabilizer-hybridization accelerator formulation for hybridization-b nucleic acid lateral flow detection platform.
Conference: 3rd International Conference on Bio-sensing Technology
Author(s): Ang, Geik Yong, Yu, Choo Yee & Chan, Yean Yean
Year: 2013

Title: A novel CTX prophage array in Kelantan cholera outbreak strain.
Conference: 4th Regional Conference on Molecular Medicine
Author(s): Yu, Choo Yee, Ang, Geik Yong & Chan, Yean Yean
Year: 2011

Title: Cholera outbreak caused by *Vibrio cholerae* O1 with atypical El Tor biotype in Terengganu, Malaysia
Conference: 16th National Conference on Medical and Health Sciences
Author(s): Yu, Choo Yee, Ang, Geik Yong & Chan, Yean Yean
Year: 2011

[√] Book(s) [1] quantity [ ] quantity

Please state
Title: Genetic analysis of CTX prophage and antibiotic resistance determinants in Vibrio cholerae O1 belonging to the atypical El Tor biotype from Kelantan, Malaysia.
Publication: InTech
Author(s): Yu, Choo Yee, Ang, Geik Yong & Chan, Yean Yean
Year: 2012

[ ] Others, please specify: ____________________

4.0 National and International Research Collaboration (involvement of research institutions role; MoU/MoA, if applicable)

a) National

NIL

b) International

NIL

How did the project contribute to expertise?

☑ PhD degrees
Please state
Name: Yu Choo Yee
Nationality: Malaysian
Area of Specialisation: electrochemical DNA Biosensor
Duration: Jan 2010 – Jan 2014

Name: Ang Geik Yong
Nationality: Malaysian
Area of Specialisation: lateral flow DNA biosensor
Duration: Jan 2010 – Jan 2014

☐ MSc degrees
Please state
Name:
Nationality:
Area of Specialisation:
Duration:

Name:
Nationality:
Area of Specialisation:
Duration: (Date Started - Date Completed)

☑ Research staff with new specialty
Please state
Name: Balqis Kamarudin
Nationality: Malaysian
Area of Specialisation: DNA Biosensor
Duration: Aug 2011 – May 2013

☐ Others, please specify: ____________________
Please state
Name:
Nationality:
Area of Specialisation:
Duration: (Date Started - Date Completed)

What are the infrastructure contributions of your project?

- [ ] New equipment
  Please state:

- [x] New/improved facility
  Please state:
  Refrigerated centrifuge

- [ ] New information networks
  Please state:

- [ ] Others, please specify: ______________________________

E. CONTRIBUTION OF THE PROJECT TO THE ORGANIZATION'S REPUTATION

How has the project contributed in increasing the reputation of your Organization?

- [ ] Recognition as a Center of Excellence

- [ ] National award
  Please state
  Type:
  Category:

- [ ] International award
  Please state
  Type:
  Category:

- [x] Demand for advisory services

- [x] Invitations to give speeches on conferences

- [x] Visits from other organizations

- [ ] Others, please specify: ______________________________

Early identification and measurement of commercial potential

echnology escription	A new approach for the detection of *V. cholerae* and cholera toxin based on the development of a multiplex electrochemical-enzyme based DNA hybridization genosensor was developed in this study. This sensor exploits the affinity of single-stranded DNA (ssDNA) for its complementary strands to detect specific sequence of target DNA and findings of this study contribute towards the development of portable analytical devices.
otential pplications	The use of gold electrode substantially simplifies the method that is used for functionalizing the electrode and this will promote the use of biosensor in laboratory for detection of nucleic acid or PCR amplicons.
rocess valuation	*The process aspects of the evaluation address the adoptability and adaptability of a new technology intc existing methods of utilization or the process that a technology is going to replace. Whether or not the technology can be adopted on a small scale before investing in major overhauls of existing processes. Anothei issue can be addressed, whether the bulk of the potential market actually possesses the technical capability tc integrate this new technology into their operations.* A prototype has been developed but it requires further research work for integration into user-friendly, automated-based technology platform such as microfluidic and microarray.
conomic valuation	*Costs implementing of the technology (for example any process changes). Outweigh costs of operation anc maintenance* NIL
arket valuation	*Identification and assessment of a market demand for the technology. Is this a technology that will be purchasec by only a few specialized users, or is the demand likely to exceed niche boundaries? Identified and comparec number of competitors/groups that are developing parallel technology in terms of effectiveness, cost, and ease of use or process integration.* Future research is required to make the prototype user-friendly before it can become commerciall viable
roposed Future Vork	[√] Up scaling of laboratory prototype [√] Development of commercial ready prototype [ ] Development of pilot plant [ ] Pre-clinical or Clinical trials/field trials [ ] Acquisition of foreign/local technology [ ] Others, please specify: <u>Brief explanation :</u> The current biosensor prototype has been developed as a proof-of-concept and further researcl work is required for integration into microarray or microfluidic system in order to make the assa platform commercially viable.

<table>
<tr><td colspan="3"><u>Project Leader</u></td></tr>
<tr><td>Date: 23/ 1 /2014</td><td>Signature: </td><td>Dr. Chan Yean Yean
Lecturer
Department of Medical Microbiology & Paras
School of Medical Sciences, Health Camp
Universiti Sains Malaysia, 16150 Kubang K
Kelantan, Malaysia.</td></tr>
</table>

Comment and Recommendation by RMC/ Institutional Research Coordinator/Committee		
Date:	Signature: Name:	Official Stamp:

Attachment:
(1.1) Milestones description/Summary of the Progress

M1: Three pairs of primer have been designed for the simultaneous identification of *V. cholerae* serogroups O1 and O139 as well as for the detection of cholera toxin (CT) gene. The fourth primer pair which functions as an internal amplification control (IAC) was incorporated in the assay to rule out false negative results. The oligonucleotide sequence of the primers is shown in Table 1. The specificity of the designed primers was checked using BLAST available at the GenBank website (http:// blast.ncbi.nlm.nih.gov/Blast.cgi).

Table 1. Oligonucleotide sequence for primers used in this study

Target Gene	Primer (5' to 3')		Product Size (bp)
IAC	LaIAC-FX	TGT AGG TTG TCA TCC ATC AG	112
	LaIAC-RL	TTC TAC TTC AAC ACA AAC AGC CTT C	
V. cholerae O1	LaO1-FX	AAC AAG TCG TTC GCC TTA TTA AC	155
	LaO1-RL	AAT GAT AGA ACC GCC GCC AAG T	
CT	La*ctxA*-FX	CAG TCC TCA TCC AGA TGA AC	221
	La*ctxA* RL	GCT CTT CCC TCC AAG CTC TAT GC	
V. cholerae O139	LaO139-FX	ACG CAC GCT TAT CAA TAC AG	289
	LaO139-RL	CGA TCC CGA ATC AAG ATC AAC TGT	

*FX: Forward excess primer; RL: Reverse limiting primer

M2: The mLATE-PCR was standardized using purified genomic DNA from *V. cholerae* O1 and O139. First,a monoplex PCR was performed to ensure that each primer pair amplifies the intended target gene as shown in Figure 1.

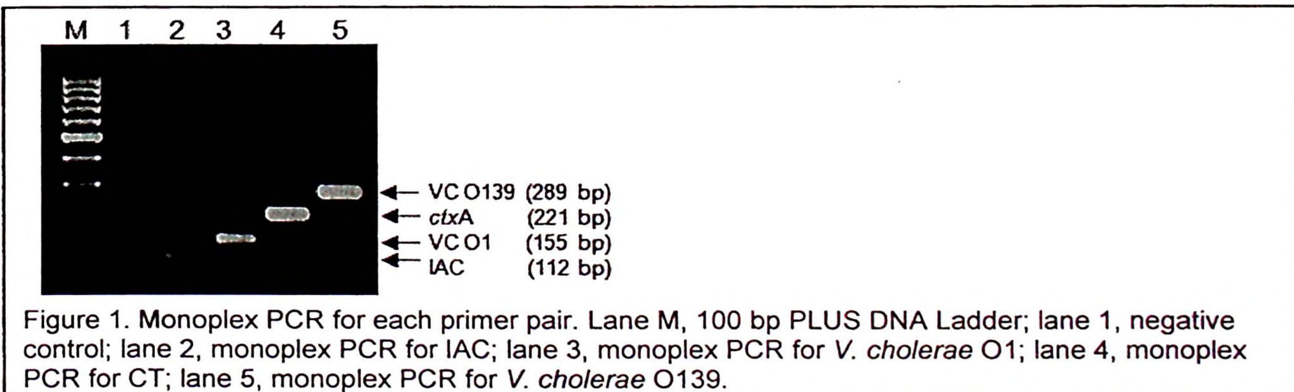

Figure 1. Monoplex PCR for each primer pair. Lane M, 100 bp PLUS DNA Ladder; lane 1, negative control; lane 2, monoplex PCR for IAC; lane 3, monoplex PCR for *V. cholerae* O1; lane 4, monoplex PCR for CT; lane 5, monoplex PCR for *V. cholerae* O139.

Then, a multiplex PCR was performed for the amplification of CT and simultaneous identification of *V. cholerae* O1 and O139 as shown in Figure 2. It can be seen in the figure that the primer pairs of *V. cholerae* O1 and O139 amplifies their respective serogroup specifically.

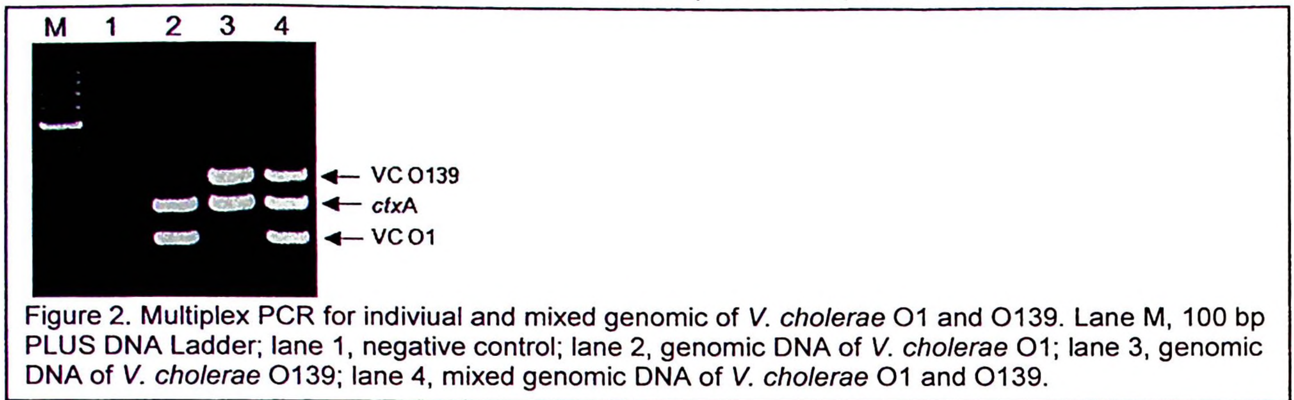

Figure 2. Multiplex PCR for indiviual and mixed genomic of *V. cholerae* O1 and O139. Lane M, 100 bp PLUS DNA Ladder; lane 1, negative control; lane 2, genomic DNA of *V. cholerae* O1; lane 3, genomic DNA of *V. cholerae* O139; lane 4, mixed genomic DNA of *V. cholerae* O1 and O139.

Various parameters of the multiplex Linear-after-the exponential (LATE)-PCR (such as the annealing temperature, concentrations of dNTPs, $MgCl_2$, *Taq* DNA polymerase) were subsequently optimized.

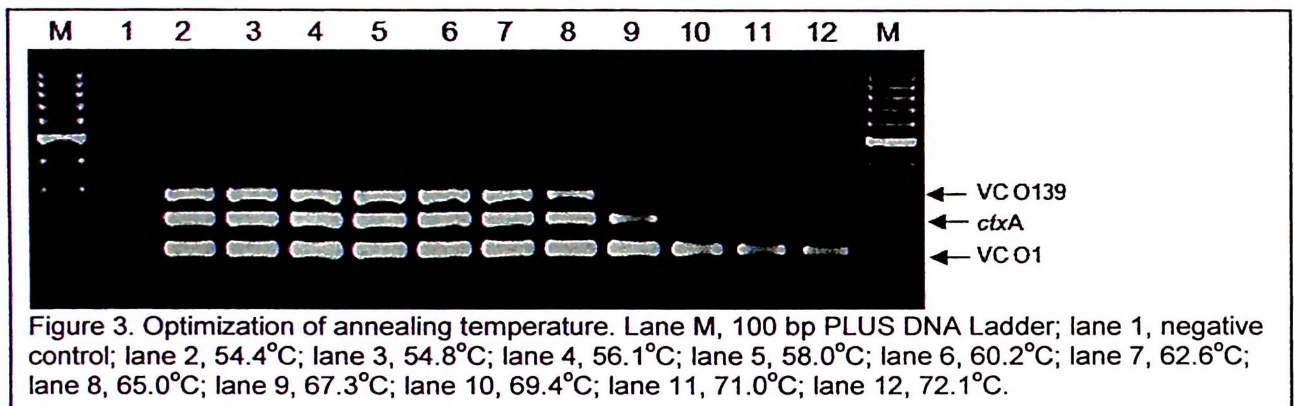

Figure 3. Optimization of annealing temperature. Lane M, 100 bp PLUS DNA Ladder; lane 1, negative control; lane 2, 54.4°C; lane 3, 54.8°C; lane 4, 56.1°C; lane 5, 58.0°C; lane 6, 60.2°C; lane 7, 62.6°C; lane 8, 65.0°C; lane 9, 67.3°C; lane 10, 69.4°C; lane 11, 71.0°C; lane 12, 72.1°C.

Selected optimal annealing temperature: 60 °C.

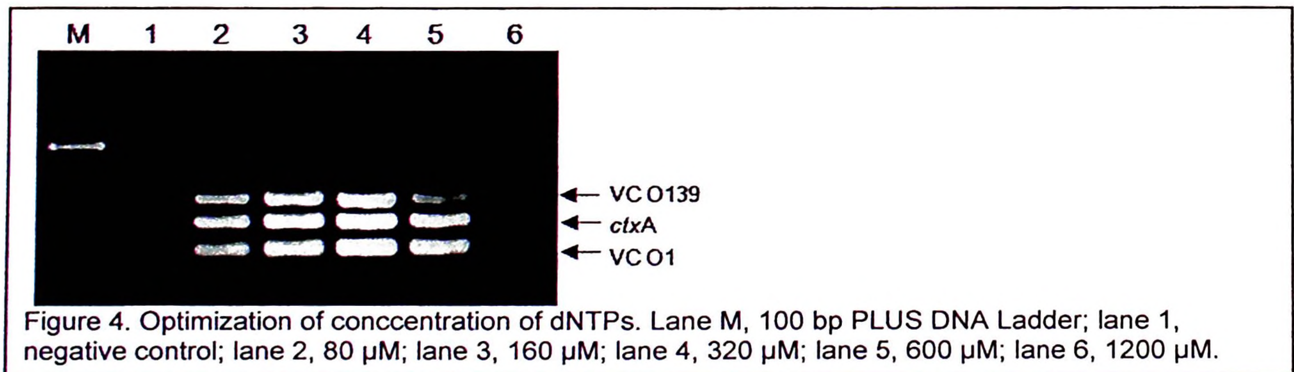

Figure 4. Optimization of conccentration of dNTPs. Lane M, 100 bp PLUS DNA Ladder; lane 1, negative control; lane 2, 80 µM; lane 3, 160 µM; lane 4, 320 µM; lane 5, 600 µM; lane 6, 1200 µM.

Selected optimal concentration of dNTPs: 320 µM.

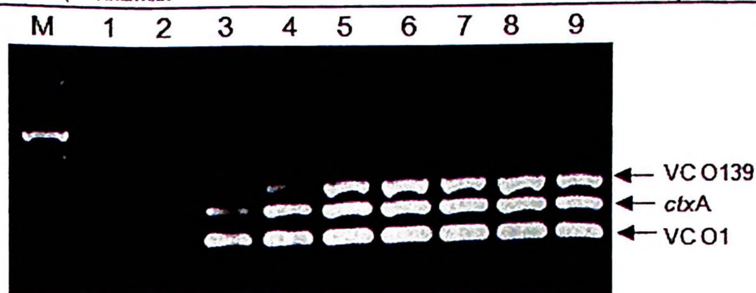

Figure 5. Optimization of conccentration of $MgCl_2$. Lane M, 100 bp PLUS DNA Ladder; lane 1, negative control; lane 2, 1.5 mM; lane 3, 2 mM; lane 4, 2.5 mM; lane 5, 3 mM; lane 6, 3.5 mM; lane 7, 4.0 mM; lane 8, 4.5 mM; lane 6, 5.0 mM.

Selected optimal concentration of $MgCl_2$: 4.5 mM.

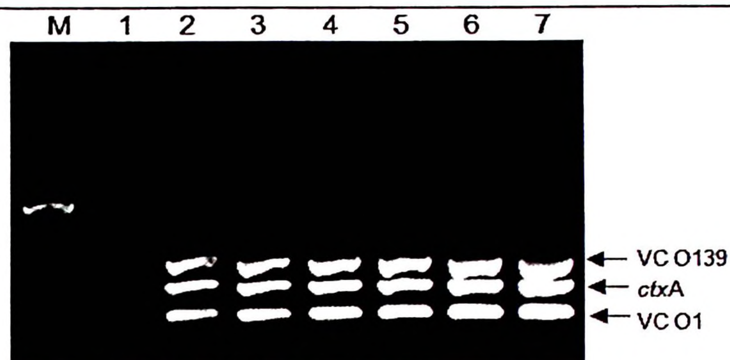

Figure 6. Optimization of conccentration of *Taq* DNA polymerase. Lane M, 100 bp PLUS DNA Ladder; lane 1, negative control; lane 2, 0.25 U; lane 3, 0.5 U; lane 4, 0.75 U; lane 5, 1.0 U; lane 6, 2.0 U; lane 7, 3.0 U.

Selected optimal concentration of *Taq* DNA polymerase: 0.75 U.

After optimizing the parameters affecting the mLATE-PCR, an IAC was incorporated into the mLATE-PCR to rule out false negative results. At various amounts of IAC template tested (0.005 ng to 0.05 ng) tested, 0.05 ng/µl was found to be optimal with minimal compromise on the sensitivity of the assay.

Below is the optimized conditions for the optimized mLATE-PCR.